STUDIES ON THE FUNCTION OF RHOPTRY NECK PROTEIN 11 (RON11) IN *PLASMODIUM FALCIPARUM* MEROZOITES AND SPOROZOITES

by

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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, Maryland
May 2022

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Abstract

Malaria is a life-threatening disease that mainly affects pregnant women and children under 5, with a mortality rate of 12 deaths per 1,000 individuals per year. Over 94% of *P. falciparum* cases occur in Sub-Saharan Africa. Eradication campaigns have been implemented that consist of vector control, chemotherapy and chemoprophylaxis. However, effective implementation of these strategies remains a challenge. The emergence of resistance to front-line antimalarials exacerbates this problem. RTS,S (Mosquirix) the first malaria vaccine shown to protect children against malaria is currently being implemented. However, vaccine efficacy wanes from ~40% in the first year to less than 30% in subsequent years. Importantly, this vaccine does not target the disease-causing forms of the parasite. Understanding the molecular mechanisms that regulate parasite invasion of host cells can provide new antimalarial targets and lead to development of novel approaches to prevent disease. Second messenger calcium (Ca2+) signaling plays an important role during merozoite egress and invasion. How the parasite senses changes in ionic concentrations such as Ca2+ is an active area of interest.

The goal of this study is to characterize the role of PfRON11, an EF-hand domain containing protein, in *P. falciparum* merozoites and sporozoites. Our recent mass spectrometry analysis of invasive merozoites identified RON11 to be in higher abundance than in non-invasive merozoites. Interestingly, a high throughput genetic screen in the rodent malaria parasite *P. berghei* identified RON11 to be essential during parasite asexual growth and subsequently also shown to be required for sporozoite invasion of hepatocytes.
In this study our aims were, 1) examine the expression and localization of RON11 in *P. falciparum* merozoite and sporozoite, 2) evaluate activity of antibodies targeting RON11 in neutralizing *P. falciparum* merozoites, and 3) test the Ca2+ binding potential of the EF hand domain of RON11.

We expressed recombinant protein corresponding to the N-terminus of RON11 in *E.coli*, generated antibodies in rats and use these antibodies to study protein expression and localization in merozoites and sporozoites. We further demonstrate that anti-RON11 antibodies can neutralize merozoites. Furthermore, we optimized expression of the putative calcium binding RON11 EF hand domain in HEK293T cells to study its ability to bind Ca2+. Lastly, we generated a dual conditional knock out/conditional knock down plasmid to replace the endogenous RON11 gene using Crispr/Cas9 – mediated homologous recombination in *P. falciparum* and attempted parasite transfections.

**Keywords:** Malaria, *Plasmodium*, Rhoptry Neck (RON) proteins, EF-hand, calcium signaling

**Primary reader:** Prakash Srinivasan

**Secondary reader:** Photini Sinnis
Acknowledgements

There are an innumerable number of people that have supported me professionally and personally that has allowed me to complete my thesis.

Firstly, I would like to thank my thesis advisor, Dr. Prakash Srinivasan, who has shaped me into a resilient and curious science. Thank you for the opportunity to work in your lab and guiding me throughout my multiple projects. Not only did I have the opportunity to learn multiple lab techniques, but also, learned to present my work and become more critical. Beyond that, I have become more resilient and confident in myself as a scientist.

I would also like to thank every single past and present member of the lab. Dr. Deepti Sarkar, Dr. Maryam Saffarian Abbas Zadeh, Sean Yanik, Zachary Berliner, Opeoluwa Adewale-Fasoro, Varsha Venkatesh and Elgin Akin. Thank you for always being available to teach me something new and answering any of my many questions. Beyond that, I would like to thank you for the constant life support and listening to me vent and laugh.

Secondly, I would like to thank my secondary reader, Dr. Photini Sinnis. Thank you for the advice and guidance you have given me on my thesis. In addition, I would like to thank Dr. Marcelo Jacobs-Lorena and his lab members for their guidance and sharing lab equipment. I would also like to thank the JHMRI insectary core, especially Godfree Miambo, for providing me mosquitoes and allowing me to proceed with my experiments. I would also like to thank Karen Griffin for all the hard work she does. In addition, Gail O’Connor for everything that you do and all the support you give. I would also like to thank Dr. Connor McMenniman for the support in the beginning of my masters program, in the midst of a pandemic. All in all, I’d like to thank the department as a whole.

I would also like to thank my ScM cohort for being a constant uplifting presence and always being so supportive. I would like to extend this to my housemates, my friends and family that have been a source of constant support in this unique period of my life. Specifically, I would like to thank my aunt Ana Isabel Ribeiro, that never doubted my abilities and always pushed me to do more than I thought I was capable.
Lastly, I would like to thank Fulbright and their team. Without them, I would not be where I am today and for that I am thankful. Beyond all the academic knowledge and training as a scientist I obtained during my master’s program at BSPH, I also learned to go through life with a sense of community and belonging and learned to be mindful and involved in the community that I am inserted in.
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**Introduction**

*Malaria Introduction – Epidemiology and burden of disease*

Malaria is an ancient disease. It is distinguished by distinct fevers, chills, and enlarged spleens. It has been dated back to 2700 BC and characterized in Chinese documents, clay tablets, and Egyptian papyri (1-3). Additionally, it was associated with swampy and crowded areas and standing water (1, 2). The disease was named Malaria by the Romans, meaning *bad air* in Italian, as it was first believed to occur due to toxic fumes (1, 2, 4). It was thought to be imported to the New World via the slave trade. It wasn’t until 1880 that Charles Laveran investigated febrile soldiers’ blood and identified what is now known to be the malaria parasite (2, 3).

Malaria occurs mainly in tropical regions of the world. Specifically, Africa and Asia are the most affected, with vulnerable groups, young children, and pregnant women suffering the most severe side effects (2, 5, 6). Symptoms of the disease include general malaise, weakness, fevers, and muscle aches (7, 8). Although these are classified as more general symptoms, in malaria-endemic countries, they are easily identifiable and diagnosed as malaria. When diagnosed and treated early, the infection can easily be treated (7, 8). When a patient remains untreated, more severe symptoms may occur that can lead to death. These include severe anemia and cerebral malaria. It is still unknown what exactly triggers these severe complications (8). Children under the age of five are most susceptible to severe disease, as they have not yet developed clinical immunity, whereas adults and older children who are exposed to repeated infections are clinically
protected. Additionally, placental malaria is a severe complication, as it may lead to maternal anemia and consequently low birth weight of the child (2, 7, 8).

According to the 2021 World Malaria Report, there were about 241 million malaria cases worldwide in 2020. Sub-Saharan Africa accounts for about 96% of *P. falciparum* cases globally. More specifically, 55% of global cases occur in Nigeria, the Democratic Republic of Congo, Uganda, Mozambique, Angola and Burkina Faso (World Malaria Report, 2021). The South-East Asia Region is also affected and accounted for about 2% of malaria cases globally. In general terms, the malaria mortality rate has increased by 12% compared to 2019 (World Malaria Report, 2021) (Figure 1).

*Figure 1. Map of the world, showing areas where malaria transmission occurs (5).*
**Malaria prevention, drugs, and vaccines**

In endemic countries, when patients present with malaria-like symptoms, they are immediately tested for malaria. The WHO diagnosis criteria include fever and the presence of the parasite in the bloodstream (9). Detection of the parasite can be done by direct microscopic observation of the parasite in a Giemsa-stained thick and thin blood smear. Rapid diagnostic tests (RDTs) can also be performed; however, these are less reliable (8, 10). Timing of diagnosis is crucial for preventing severe disease.

For people traveling to malaria-endemic counties, preventative strategies must be implemented. These include both chemoprophylactic agents and measures to reduce mosquito exposure. Chemoprophylaxis agents vary depending on individual risk (11). In addition, individuals should avoid outdoor activity at night to prevent *Anopheles* mosquitoes from biting, use long-sleeved shirts and pants, use insecticide-treated bednets (ITNs), and insect repellents containing 50% DEET *N,N*-diethyl-m-toluamide (11).

For residents in malaria-endemic areas, different prevention and control efforts have been implemented. However, these are limited by financial resources, as malaria-endemic areas typically overlap with developing countries (9-12). Preventative measures include vector control and chemotherapy. Vector control includes the use of insecticides and larvicides, destruction of breeding grounds, indoor residual sprays (IRS), and use of ITNs. Limitations in vector control strategies include their inappropriate use and monitoring, mosquitoes may alter their behavior and may gain resistance to insecticides (9-12).
In a second approach, chemotherapy has also been shown to be an effective strategy to reduce the incidence of malaria cases when administered to those at high risk of severe malaria, such as children and pregnant women. However, these are costly approaches and when disrupted, individuals may lose immunity, leading to rebound (9-12). Chemotherapy, however, is used when a patient is diagnosed with malaria. Intermittent preventative treatment (IPT) or seasonal malaria chemoprevention (SMC) are approaches used to treat malaria in endemic areas. IPT is a strategy that involves administering a full curative dose of an antimalarial to vulnerable asymptomatic individuals (children under 5 and pregnant women) (13). SMC consists of intermittent full malaria treatment to children under 5 (14). However, the parasite may develop resistance (9).

Lastly, malaria vaccines are being studied to eradicate the disease. Ideally, the vaccine should protect against both *P.falciparum* and *P.vivax* and have long-lasting protection and high efficacy (9). These need to be designed keeping in mind the target population (children below the age of 5). Vaccines targeting different stages of the parasite life cycle (pre-erythrocytic, blood stage and transmission blocking) may be needed to confer high efficiency protection (8).

Interestingly, individuals living in malaria-endemic countries develop naturally acquired immunity that protects against disease. This depends on many factors such as region, age and, number of exposures (12). Typically, children in malaria-endemic regions are born with malaria-acquired immunity that is transferred through maternal antibodies. However, this passive immunity is lost within 6-9 months. After this period, until about five years of age, children are most susceptible to infections. When repeated exposure occurs, individuals aged five and above develop naturally acquired immunity. When removed from such environment, the immunity can
also be lost (10). In addition, hereditary factors may also contribute to protection against the parasite. This includes, for example, the sickle cell trait that is shown to protect against disease (10). Another example is the Duffy blood-group antigen, which is required for *P. vivax* invasion in RBCs (10). Absence of this antigen in many parts of Sub-Saharan Africa is thought to protect against *P. vivax* (10). Lastly, other hemoglobinopathies (e.g., thalassemia) have also been shown to offer protection against infection (10).

Recently, the first malaria vaccine received endorsement by the World Health Organization (WHO) and is recommended to children living in malaria-endemic countries. This is the RTS,S vaccine, made up of the circumsporozoite protein (CSP) combined with hepatitis B surface antigen (12). Its efficacy was established in 2014 in a Phase 3 clinical trial done in 15,459 children in malaria-endemic regions (Burkina Faso, Gabon, Ghana, Kenya, Malawi, Mozambique, and Tanzania). This showed that children who received four doses had a 39% reduced risk of infection and 29% less severe disease. Currently, four dosages of the vaccine are recommended for children aged five months onwards (10, 12). This vaccine was first developed in 1987, and only in 2021 was it approved, highlighting how difficult it is to develop a malaria vaccine.

**Lifecycle of the Parasite**

Although the parasite was identified in 1880, only in 1897 did Ronald Ross identify the mosquito as the vector when he saw the parasite in the stomach of an *Anopheles* mosquito who fed on an infected patient. A year later, Bignami and Grassi proved that the parasite was mosquito
transmitted. Understanding the lifecycle of the parasite was a long process that involved multiple scientists, as it involves three players: the parasite, the vector, and the human host (2, 4, 15-17).

Today, it is known that malaria is caused by the protozoan pathogen of *Plasmodium* species, which is an obligate intracellular parasite (9, 18). There are five different *Plasmodium* species that affect humans. However, the most common are *Plasmodium falciparum* (*P. falciparum*) and *Plasmodium vivax* (*P. vivax*) (9, 18). *P. falciparum* is the dominant species in sub-Saharan Africa, whereas *P. vivax* is dominant in Southeast Asia and South America (5, 9, 18). In this paper, *P. falciparum* will be the main form of malaria described.

The parasite is spread when an infected female *Anopheles* mosquito feeds on a human host. During the blood meal, the mosquito injects the sporozoite stage of the parasite into the skin. It is thought that they take 1-3 hours to exit this site and rely on processes such as gliding motility to penetrate a blood vessel and enter the bloodstream (19-21). Gliding motility uses an actin-myosin motor with the aid of proteins such as thrombospondin-related anonymous protein (TRAP) that bridges binding of actin to myosin and facilitates gliding (18). Of the sporozoites injected, a fraction invades the hepatocytes, whereas the majority drain into the lymph node and are removed (18).

The sporozoites that invade the hepatocytes do so by the formation of a parasitophorous vacuole membrane (PVM), where they multiply (7, 18). In hepatocytes, sporozoites replicate as merozoites. One sporozoite gives rise to about 40 thousand merozoites (9, 18). It is also important to mention that *P. vivax* lifecycle includes a dormant liver stage termed hypnozoites that may remain for years before re-infection. Merozoites then rupture and enter the bloodstream,
where they are able to invade red blood cells (RBCs). This is known as the blood stage of the parasite and is also known as asexual reproduction. Merozoites produced within the infected RBCs are subsequently released into the bloodstream, allowing for a new cycle of erythrocyte invasion. Parasite growth within RBC takes 24-72 hours depending on the Plasmodium species and is associated with the symptomatic stage of disease (5, 9, 18). During the blood stage, the parasite releases multiple proteins into the host cell cytoplasm that consequently lead to changes in the cell surface and adhesion (5, 9, 18, 22). One major surface antigen is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is encoded by the var gene family. This family contains 60 different genes, classified into three families. The variability within the gene contributes to parasite polymorphism that allows them to evade the human immune system. PfEMP1 family proteins mediate cytoadherence of infected RBCs, allowing them to bind to each other and to the endothelial vasculature. This is thought to contribute to cerebral and placental malaria (5, 9, 18, 22).

A small percentage of parasites in the blood stage develop into gametocytes. These can be ingested by the mosquito when they take a blood meal. In the mosquito, male and female gametes fertilize to form zygotes that will then develop into ookinetes. Upon traversal of the midgut epithelium, the ookinetes transform into a cyst. Thousands of sporozoites formed within each oocyst rupture and are transported into the salivary glands of the mosquito and are further released during blood-feeding, starting a new human cycle. This process takes 7-10 days after the mosquito takes a blood meal (5, 9, 18, 22). The cycle is depicted in figure 2.
Merozoite invasion

A) Merozoite structure

*Plasmodium* species belong to the phylum Apicomplexa, which compromise different parasites such as *Toxoplasma, Cryptosporidium*, etc. This phylum is characterized by having an apical structure and containing apical organelles, such as the micronemes and rhoptries near the tip of the apex (15, 23-25).

The merozoites are shown to have a pear-shaped structure, having a broad posterior end and a thinner anterior apical end. These are also the smallest of all the parasite stages (15, 23-25). At
its apical pole, it contains polar rings, which are bound by a triple membranous structure. These consist of the plasma membrane and two other membranes that together form the inner membrane complex (IMC) and are shown to harbor proteins essential for invasion and motility of the parasite (15, 23-25).

The apical organelles include rhoptries and micronemes, which are shown to contain proteins essential for host cell invasion. More specifically, merozoites are thought to contain adhesins necessary for erythrocyte binding. Merozoites contain two pear-shaped rhoptries attached to the apical pole by a split duct. The micronemes are small and elongated vesicles around the rhoptry duct, and merozoites contain around 40 of them. These are shown to be released after binding to the RBC and allow the formation of the parasitophorous vacuole (PV). In addition, merozoites also contain dense granules that contain proteins essential for establishing a translocon on the PVM and allow parasite proteins to be exported into the RBC cytoplasm (15, 23-25). The structure of the merozoite is depicted in figure 3.

![Figure 3](image.png)

*Figure 3.* Illustration of a Plasmodium merozoite and the apical organelles that store proteins involved in invasion (24).
B) Merozoite invasion of RBCs

Merozoite invasion can be characterized in 4 phases: 1) Initial attachment of the merozoite to the surface of the RBC, 2) Apical reorientation, 3) Junction formation and 4) entry into the RBC using its actin-myosin motor. The overall process is relatively quick, taking ~30 seconds after the initial interaction (15, 23-25).

During the first phase, the merozoite encounters an erythrocyte. This attachment is reversible, and the parasite is not yet committed to invasion. This initial contact is shown to be mediated by glycosylphosphatidylinositol (GPI) merozoite surface proteins (MSPs) (15, 23-25). MSP1, the most abundant protein on the merozoite surface and is shown to be involved both egress and in invasion by forming a multiprotein complex with other peripheral proteins (15, 23-25).

After initial attachment, the merozoite reorients, allowing the apical organelles to line up at the apex and come into closer proximity with the erythrocyte membrane (15, 23-25). The initial tight interaction is mediated by the Duffy binding-like (DBL) and reticulocyte binding protein (PfRH) family (15, 23-25). Engagement of these proteins with their receptors allows for a deformation of the erythrocyte surface, facilitating invasion. This deformation is shown to be induced by PhRH5 binding to host receptor basigin (15, 23-25). RH5 is the smallest Rh family protein and has been shown to be essential for invasion. Binding to its receptor leads to an increase of Ca^{2+} concentration and further alteration of the RBC cytoskeleton (26). Subsequently, rhoptry neck proteins (RONs) such as RON2 are secreted by the parasites and inserted into the erythrocyte membrane. Apical membrane antigen 1 (AMA1), a micronemal protein secreted on to the merozoites surface then binds to RON2 and commits the parasite for invasion by forming a
moving junction (MJ) (Fig 4, (15, 23-25)). This is a unique adaptation where both receptor and ligand are provided by the parasite to mediate successful invasion of the host cell. The MJ is also thought to be coupled with the actin-myosin motor, which allows the parasite to be propelled into the newly forming PV. The actin-myosin motor is thought to be linked to adhesins through their cytoplasmic tail linking them to the inner membrane complex (IMC) and together allows for parasite motility. Multiple proteins are involved (MTIP, GAP-45, GAP-50), and initiation is triggered by second messenger intracellular signals (eg., Ca²⁺) (15, 23-25).

![Figure 4. Steps of Merozoite invasion into a RBC (45).](image)

**Calcium signaling and Rhoptry proteins**

**A) Calcium signaling**

In eukaryotic organisms, intracellular signaling is essential in regulating their ability to respond to stimuli. Intracellular signaling commonly relies on second messengers such as calcium, which
regulate a wide range of processes such as protein secretion, motility, gene expression, and cellular development (27, 28). Often, this process is tightly regulated and maintained at low levels in the cytosol of eukaryotic cells, as Ca\(^{2+}\) is actively pumped into the extracellular space. When stimulated, Ca\(^{2+}\) is signaled from internal stores and released, leading to binding to effector proteins and downstream signaling cascades, which regulate cellular processes. This is a relatively quick process, and once terminated, Ca\(^{2+}\) levels are returned to their basal state (27-29).

Calcium ions are stored in specific organelles such as the endoplasmic reticulum (ER) and in acidic organelles such as the acidocalcisomes and food vacuole (FV) (27, 29). In eukaryotic cells, Ca\(^{2+}\) is maintained at a concentration of \(~100\text{nM}\), referred to as the basal state. Cells are stimulated in response to extrinsic factors such as hormones, neurotransmitters, antibodies, pH change, temperature, or even mechanical or electrical stimuli. Stimulation leads to an increase in Ca\(^{2+}\) levels and further signaling. Once stimulation is withdrawn, cells restore to their basal state (30).

Calcium ions can bind 4-12 oxygen atoms, but typically 6-8 oxygen atoms surround Ca\(^{2+}\) ions in a pentagonal bipyramid shape (31). One of the best chelators of Ca\(^{2+}\) ions is the EF-hand domain, found in multiple proteins. The EF-hand domain is a helix-loop-helix motif characterized by 12 residues. They form a loop, where calcium and magnesium bind. It is found in small and large proteins and is usually found in pairs (31, 32). When Ca\(^{2+}\) ions bind, the EF-hand proteins open and expose a hydrophobic surface that can bind the target sequence (32).
Other common calcium sensors include calmodulin, C2 domains and, phosphatidylinositol 4, 5 bisphosphates (PIP2). Calmodulin is a small adaptor protein that contains an EF-hand. When Ca$^{2+}$ bind, calmodulin undergoes a conformational change, where the hydrophobic surface is exposed, triggering Ca$^{2+}$ sensor activity and binding to its target (31). Additionally, C2 is an amino acid segment that binds to 2-3 Ca$^{2+}$ ions. It is usually a signal transducer protein that triggers the translocation of proteins to specific regions, where they can bind to substrate and relay signal (31). Lastly, PIP2 is found on the plasma membrane, which, when hydrolyzed, leads to inositol 1,4,5-trisphosphate (IP3) production and activation of Protein Kinase C (PKC) (30-32). These are second messengers that trigger the release of calcium. IP3 essentially binds to a receptor on the ER, allowing Ca$^{2+}$ ions to flow from the ER lumen to the cytosol (30, 33). The flow of Ca$^{2+}$ ions occurs via membrane channels. These include store-operated calcium channels (SOCEs), plasma membrane Ca$^{2+}$-ATPase (PMCA) and sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA) pumps (Figure 5) (29, 31).

![Figure 5. Calcium gradients used for signaling (31).](image-url)
B) Calcium signaling in Plasmodium

In *Plasmodium*, Ca$^{2+}$ transport is not as well characterized. The *Plasmodium* genome is thought to encode about 30 proteins that contain putative Ca$^{2+}$ binding domains, with the EF-hand domain being a common Ca$^{2+}$ chelator and has been identified in 20 different proteins (27, 31). Three main Ca$^{2+}$ binding protein (CaBPs) families include the calmodulin (CaM) family, the calcineurin B-like (CBL) family, and the Ca$^{2+}$-dependent protein kinases (CDPKs) (27, 29).

One calmodulin protein has been identified in the *Plasmodium* genome. This protein has been shown to be localized to the cytoplasm during the mature stage of the intraerythrocytic cycle and at the apical end of merozoites (27, 29). Protein kinase B (PKB) has been shown to interact with PfCaM, and this interaction is shown to be calcium-dependent (34). Additionally, the CDPKs that contain a Ca$^{2+}$-binding domain (27). Seven different CDPKs have been identified in *P. falciparum*. The structure is conserved among species, having a variable N-terminal domain, fused to a catalytic kinase domain, a CDPK activation domain (CAD), and a Ca$^{2+}$-binding domain (27).

More broadly, a study has been done to characterize the role of Ca$^{2+}$ in the *Plasmodium* lifecycle (35, 36). It has been demonstrated that Ca$^{2+}$ plays a role both during development of rings to trophozoites and during invasion of merozoites. Additionally, Ca$^{2+}$ fluctuations have been seen throughout the parasite lifecycle (35, 36). A study by Weiss et al in 2015 studied the role of extracellular calcium on merozoite invasion (35). They labelled RBCs with Fluo-4, a membrane permeable calcium-sensitive dye, and added mature schizonts to the culture and studied the calcium localization. They found that Ca$^{2+}$ signals displayed a punctate pattern and interestingly, the signal mostly localized to the apical tip of the merozoite. This indicates that perhaps there in
an influx of calcium ions from the RBC to the merozoite during the process of invasion (35). The exact mechanism of influx and proteins involved in this process is unknown, however, it is interesting to see the essentiality of Ca\(^{2+}\) signaling in this process.

The signaling process is similar between eukaryotic organisms. However, there are differences in signaling molecules and particular lineage-specific adaptations (27). In the case of Plasmodium, the lifecycle is thought to be regulated by fluctuations in calcium levels. However, they are distantly related to commonly used model organisms that have been used to study signaling pathways and are shown to have distinct features (27, 29). Therefore, not much is known about calcium signaling in Plasmodium parasites. In addition to the differences in a model organism, about 60% of the parasite genes are not annotated, making it challenging to make computational predictions of Ca\(^{2+}\) signaling molecules. Furthermore, some Ca\(^{2+}\) signaling molecules found in eukaryotes are not identified in the Plasmodium genome (27). Currently, most of what is known about Ca\(^{2+}\) signaling in Plasmodium relies on pharmacological studies, which use small compounds to study signaling pathways. This, however, is also challenging, as the targets of these molecules are not identified, making it difficult to interpret the findings (27). More recently, genetic tools that allow for conditional manipulation of genes are making it possible to study Ca\(^{2+}\) signaling in malaria parasites. These have revealed their importance in parasite maturation, invasion, gliding motility, and sexual stage development. However, there are still many gaps in understanding the role of calcium signaling in these parasites (27-33, 37-40). Understanding these molecular pathways involved in Ca\(^{2+}\) signaling could help identify new antimalarial targets to prevent both infection and disease.
C) Rhoptry and rhoptry neck proteins

More than 30 proteins have been classified as rhoptry proteins in *P. falciparum*. Several proteins have been shown to localize to the elongated part of the rhoptry organelle called the neck, referred as rhoptry neck proteins (RONs). The rhoptry neck fuses with the parasite plasma membrane upon schizont rupture, allowing secretion of multiple rhoptry neck proteins (RONs) (41, 42). Both the merozoites and sporozoites contain this rhoptry structure, however, they differ in shape and number (41, 42).

These RON proteins are highly conserved among apicomplexan parasites (15, 43-45). A study by Ishino et al. selected the 12 most expressed merozoite rhoptry proteins and studied their expression in sporozoites (46-48). They were able to demonstrate that most of the rhoptry proteins expressed in merozoites are also expressed in sporozoites, with the exception of RhopH complex proteins that localize to the rhoptry bulb and are involved in nutrient transport (47, 48). As they are expressed in both merozoite and sporozoite these proteins are likely to have shared function that are important for invasion of mosquito salivary glands and in the mammalian host, RBCs and hepatocytes.

Besides the RON2 described previously, many other RON proteins are discharged prior to invasion and may play a role in tight junction formation (49). RON2, RON4, and RON5 have been shown to be discharged as a complex and are inserted into the host cell membrane and interact with proteins on the parasite surface as AMA1, allowing for invasion (40, 47-51). These proteins are also expressed in the apical tip of the sporozoites, suggesting that RON proteins may also play a role during sporozoite invasion of mosquito salivary glands and/or hepatocyte (47, 48). RON4
has also been shown to have an essential role in sporozoite invasion of hepatocytes in *P. berghei* (52). While many RONs appear to be essential other such as RON12 appear to be non-essential (51).

Additionally, multiple proteins are involved in mediating rhoptry biology (35, 36). These include the cytosolically exposed rhoptry-leaflet-interacting protein 1 (CERLI1), rhoptry apical surface protein 1 (RASP1), CERLI1 and, FERLIN2 (41, 42). CERLI1 and CERLI2 are thought to localize on the cytosolic side of the rhoptry in *P. falciparum* parasites and are thought to play a role in secretion of rhoptry proteins prior to invasion (41, 42). Interestingly, FERLIN2 belongs to a C2-domain containing protein group and is shown to be essential for PVM rupture in *P. berghei* (41, 42). Another protein essential in signaling and also involved in rhoptry secretion is EBA175. This protein is translocated to the surface of the parasite plasma membrane upon increase of Ca\(^{2+}\). It then binds to glycophorin A on the RBC surface, stimulates rhoptry protein secretion and, restores Ca\(^{2+}\) to basal levels (41, 42). As calcium signaling is essential for egress and invasion of multiple host cell types such as RBCs and hepatocytes, understanding the function of proteins involved in this process could lead to the development of new antimalarial approaches that targets multiple parasite stages. In this study, a search of the *Plasmodium* database (PlasmoDB), identified RON11, a candidate calcium binding rhoptry protein. Protein expression and localization in *P. falciparum* merozoites and sporozoites and during merozoite invasion of RBCs were studied.
Aims

The overall goal of this study was to characterize the role of the EF-hand containing rhoptry neck protein 11 (RON11) in *P. falciparum* merozoites and sporozoites. First, we expressed recombinant protein corresponding to the N-terminal region of PfRON11 in *E.coli*. Protein was purified by affinity (Nickel) and size exclusion chromatography (SEC). Antibodies were raised against recombinant PfRON11 protein in rats and used to study its expression in *P. falciparum* blood stage and salivary gland sporozoites. We evaluated the activity of antibodies targeting RON11 to neutralize *P. falciparum* merozoites. Attempts to conditionally knockout PfRON11 in blood stage parasites were made to evaluate the function of PfRON11 in calcium signaling during merozoite egress/invasion. Lastly, a recombinant protein corresponding to the C-terminus EF-hand domains was expressed to study its ability to bind Ca^{2+}.

Methods

*Database search to identify calcium binding proteins (CaBPs) and selection of rhoptry neck protein 11 (RON11)*

A search of the *Plasmodium* database (PlasmoDB) for *P. falciparum* calcium binding domain proteins containing a predicted EF-hand (excluding rifin and emp1 gene family) or C2 domains identified 62 proteins. Of these, 29 proteins have evidence of protein expression in mature asexual stage parasites (schizonts and/or merozoites) as defined by mass spectrometry identification of at least 2 unique peptides. Fifteen (15) were predicted to be essential (piggyback insertion mutagenesis index score <0.4) in *P.falciparum* and only 7 had mass spectrometry evidence for expression in both merozoites and sporozoites. We excluded 6 proteins based on
information from previous studies as not likely to be involved in rhoptry function (see results). RON11, is a multipass membrane protein containing two EF-hand domains at its C-terminus and was selected for analysis as candidate protein involved in rhoptry secretion.

*Modeling the structure of the RON11 EF hand domains*

A search of the Protein Data Bank (PDB) using the amino acid sequence corresponding to the predicted PfRON11 EF-hand domains identified the EF hand domains from PfCDPK2 (22.4% identity) and troponinC (15.1% identity). These domains were used to generate a homology model using Protein Homology/analogY Recognition Engine V 2.0 (Phyre2) (53).

*Recombinant RON11 protein expression, purification and antibody generation*

*Protein expression*

An *E.coli* expression plasmid was first generated by cloning a codon optimized DNA fragment corresponding to the N-terminus of RON11 (amino acids 32-442) into the pMAL vector plasmid backbone containing maltose binding protein (N-terminus MBP tag). The MBP protein is known to enhance protein solubility and allows for purification using amylose resin. The plasmid backbone was digested using BamHI enzyme and left at 37.0°C for 3 hours, generating a linear plasmid. After verification by agarose gel electrophoresis that the digestion has occurred, the plasmid was inserted by Gibson assembly, as the RON11 insert contained a 20-base pair overhang necessary for the reaction to occur. This reaction occurred at 50°C for 60 minutes following the
NEBuilder HiFi DNA Assembly Protocol from New England BioLabs. In the reaction, 50ng of the linearized vector was mixed with the insert at a 1:2 vector:insert ratio, ligated using the NEBuilder HiFi DNA Assembly Master Mix, generating a RON11 EF-hand-Maltose binding protein (MBP) fusion protein in the pMAL vector. The plasmid backbone also contains a lactose operon (lac operon), which may be used for transport and metabolism in *E.coli*. This can be used to control gene expression and it was used to induce protein expression, better explained below (54, 55). In addition, the constructs contained 6x Histidine tags, with the goal of purifying the protein using nickel resin, which strongly binds to histidine (Figure 6).

**pMAL-RON11-MBP fusion plasmid**

*Figure 6. Plasmid map of pMAL-RON11- MBP fusion.*

After the plasmids were generated, they were transformed in BL21 DE3 competent cells and further selected on Ampicillin (Amp) positive LB agar plates. The colony was then cultured in LB Broth containing 100μg/mL ampicillin, overnight at 16°C in an incubator-shaker at 225rpm. As the plasmid backbone contains the Lac operon expressing system, the addition of IPTG induces the expression of RON11. The protein expression was induced with IPTG (0.4mM) 16º overnight. When IPTG is added, it binds to the lac repressor, preventing inhibition of the lac operon (56, 57). The cells were harvested by centrifugation at 17500rpm for 15 minutes in an ultracentrifuge and the pellet was then frozen at -70°C. The pellet was resuspended in lysis buffer containing 300mM NaCl, 10mM imidazole, 500mM NaH2PO4 and 1μM PMSF. In order to increase the solubility of
the expressed protein 10mM BME, and 0.1% Tween20 were added to the lysis buffer. Ultra-pure lysozyme was then added to this solution to give a final concentration of 1mg/mL and further put on a sonicator 4x2 minutes, until the sample was no longer viscous. This was then centrifuged for 15 minutes at 17500 rpm at 4°C (56, 57). The samples were run on an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and assessed by Coomassie staining and western blot using an antibody for the 6x Histidine tag to verify recombinant RON11 expression. The proteins ran on the gel were transferred to a 0.2um PVDF membrane, which was first incubated in blocking buffer (1x TBS, 0.1% Tween-20, 2% skim milk) for one hour in an orbital shaker. Then, the membrane was incubated for overnight at 4°C, with a 6x-His Tag Monoclonal Antibody at a dilution of 1:500. The membrane was washed 3 times with wash buffer (1x TBS, 0.1% Tween-20) for 5 minutes each. It was then incubated for 1 hour using the HRP-Goat Anti-Mouse IgG secondary antibody that was diluted 1:1000 in blocking buffer. Lastly, it was incubated with Supersignal West Pico PLUS Chemiluminescent Substrate and visualized and captured on a Syngene G:Box imager.

Protein purification

After verification by western blot that the protein has been induced and expressed, it was further purified using fast protein liquid chromatography (FPLC). Purification was done using an Immobilized-metal affinity chromatography (IMAC) column and CHT Ceramic Hydroxyapatite. The IMAC column contains metal ions, Ni^{2+} and Cu^{2+}, which bind strongly to the His tag of the RON11 EF-hand protein (58). The column was first equilibrated with 25mM imidazole in 20mM Sodium Phosphate Buffer pH 7.4, 300mM NaCl, at a flow rate of 2mL/min. The sample was then passed through at a flow rate of 1mL/min. A wash was performed using a 1ml/min flow rate and
53.5mM imidazole in the buffer described above. Lastly, the sample was eluted at a flow rate of 1mL/min using a stepwise gradient and the flow-through was collected in 0.6ml fractions. The FPLC chromatograph was used to select for fractions containing protein based on a positive A280 reading. These fractions were collected and run on an SDS page. Those lanes then contained protein highly specific for PfRON11-MBP were chosen. Each highly specific elution was then combined and added to a Millipore 3k cutoff column. The sample was run for 30minutes concentrating to a volume of 0.5ml. Then, 5ml of PBS with PMSF, BME, and Tween20 were added to the column. This step was repeated three times in order to allow for a near complete buffer exchange to replace the imidazole in the solution. This solution was run a second time on an IMAC column. The same settings were used as the first IMAC run. Using the A280 readings on the FPLC, we again selected for elution steps containing protein and ran each on an SDS-PAGE. Those samples with high purity were then combined, run on a 3k Millipore column, allowing for buffer exchange and concentration to a total volume of 0.5mL. In order to further purify the protein, we used a Bio-Scale Mini Cartridge, CHT Type II column compatible with the FPLC system. A linear phosphate gradient was used from 5mM to 500mM with a flow rate of 4ml/min and fractions of 2ml. A280 readings were used to identify elution fractions containing protein. These fractions were then run on SDS-page, and the four most pure fractions were combined and used for subsequent immunizations (59).

*Antibody generation and IgG purification*

Two rats were immunized subcutaneously with 0.025mg of recombinant PfRON11-MBP in Addavax adjuvant. Immunization were performed 3 times in two week intervals. Two weeks after
the final immunization, the rats were euthanized by cardiac puncture, and the whole blood was
collected (Figure 7). Blood was left for two hours at 4°C allowing for clotting while decreasing cell
lysis. Blood was then spun for 30min at 4000rpm to collect serum.

IgG was purified from the serum using a Protein G Sepharose column (60, 61). Briefly, the column
was washed and equilibrated with binding buffer before the addition of serum diluted in binding
buffer. After washing the column with 10 column volumes binding buffer, the sample was eluted
using elution buffer (pH 4) and immediately neutralized with Tris buffer (pH 9). The purified IgG
was dialyzed against incomplete RPMI and antibody concentration was measured using
NanoDrop1000 Spectrophotometer or Qubit Fluorometer.

![Figure 7](image)

*Figure 7. Immunization timeline to generate RON11-MBP antibodies.*

**RON11 EF-hand expression**

**Plasmid design**

A mammalian cell expression plasmid was designed, in which a codon optimized RON11 EF-hand
domain (amino acids 847-1041) was inserted into the pcDNA3.1 plasmid backbone (Figure 8). The
plasmid backbone was first digested using restriction enzymes NheI and ApaI at 37°C for 5 hours.
It was then run on an agarose gel and purified using the Monarch DNA Gel Extraction kit. As the synthetic RON11 insert contained 20bp overhangs for the plasmid backbone, a gibson assembly was done to piece them together. This was then transformed in XL-10 Gold Ultracompetent cells and plated on kanamycin positive LB agar plates. Colonies were then selected and inoculated in LB broth containing 100ug/mL kanamycin overnight at 37°C in an incubator at 225rpm. The plasmids were then purified, and the concentration obtained, and sanger sequenced, as described above.

In addition, this plasmid contains a cytomegalovirus (CMV) enhancer-promoter and a bovine growth hormone (BGH) polyadenylation signal and transcription termination signal. CMV promoter allows for high-level protein expression and BGH signal enhances mRNA stability (71).

PcDNA RON11 EF-hand plasmid

![Plasmid map](image)

Figure 8. Plasmid map pcDNA-RON11_EF-hand that contains a CMV enhancer, bGH polyA signal, 6xHis, Tev site and a lac promoter. The cytomegalovirus (CMV) enhancer-promoter allows for high-level protein expression and the bovine growth hormone (BGH) polyA signal enhances mRNA stability. Additionally, the 6xHis tag allows for purification using the nickel resin column.

**HEK293T transfection and protein expression evaluation**

Human Embryonic Kidney 293T (HEK293T) cells were thawed and maintained in DMEM/High Glucose media containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin Streptomycin. The cells were maintained in vented tissue culture flasks at 37°C in 5% CO₂. Once the cells reached 70-80% confluency, they were passaged to a new flask.
Transfections were done once the cells reached 70-80% confluency. The jetOPTIMUS transfection protocol was followed. Prior to starting the transfections, the media of the flasks was replaced with new media, gently without disturbing the confluency of the cells. In an initial phase, different concentrations of the plasmid were tested (1ug, 2ug and 4ug) on a 5ml transfection. The plasmid was first diluted in the jetOPTIMUS buffer and further, the jetOPTIMUS reagent was added. This was mixed properly and let to rest at room temperature for 10 minutes. Then, the transfection mixture was added to the culture flask and placed in an incubator at 37°C in 5% CO₂ for 48 hours. Then, the media and cells were collected and further analyzed to confirm protein expression. The cells were resuspended in 150ul lysis buffer (PBS, 0.1% triton-X, 1mM PMSF), vortexed and kept on ice for 30 minutes with intermittent vortexing. The samples were then spun down and the supernatant was collected. The supernatant (50ul) was run on a gel to detect protein expression by Coomasie and SDS-PAGE. Protein expression was evaluated by an SDS-PAGE and confirmed by western blot, as described above. The primary antibody used was anti-his at a 1:500 dilution and it was left at 4°C overnight. After washing, the secondary antibody was incubated for 2 hours at room temperature. The antibody used was anti-mouse at a 1:1000 dilution.

It was noticeable that 2ug of DNA was the optimal concentration for transfections and these were then upscaled to 20ml transfections. The same protocol was followed; however, 15ug of DNA was used.
TnT SP6 High-Yield Wheat Germ Protein Expression system

In an attempt to express the RON11 EF-hand proteins, the TnT SP6 High-Yield Wheat Germ Protein Expression system was tested. This system contains all components necessary to initiate transcription and translation directly from a genetic template.

First, a PCR was performed using the PcDNA-RON11_EF-hand as a template. Primers were designed in which the SP6 promoter and T7 terminator were added onto the RON11 EF-hand. A clear product was obtained, which was then used for the translation protocol.

The translation protocol consisted of a 50ul reaction, containing 30ul of the TnT SP6 High-Yield Wheat Germ Master Mix, 8ul of the PCR-generated template and nuclease-free water to make a final volume of 50ul. This was then left at 25°C for 2 hours and the results were analyzed by an SDS-PAGE (explained above).

Protein purification

We observed that the HEK293T transfections produced a cleaner result, and this method was further upscaled and used for protein purification. A his-tag pulldown was performed. The sample was diluted in IMAC binding buffer (PBS, 25mM imidazole, 1mM PMSF, BME, Tween-20) and 25ul of pre-washed nickel magnetic beads was added to the sample and left on a rotator overnight at 4°C. The following day, the beads were washed 4x with IMAC wash buffer (PBS, 25mM imidazole, 1mM PMSF, BME, Tween-20) and further eluted using 35ul IMAC elution buffer (PBS, 500mM imidazole, 1mM PMSF, BME, Tween 20). This was then run on an SDS-PAGE, using the same antibodies described above.
*P. falciparum* culture

*P. falciparum* cultures were thawed from cryopreserved malaria infected RBCs. The vials was rapidly thawed in a 37°C water bath and further 0.2 volumes of 12% NaCl followed by 9 volumes of 1.6% NaCl was added dropwise. This was then centrifuged at 2000rpm for 3 min and the pellet was then resuspended in complete medium (RPMI/HEPES medium with 5% Albumax) at 2% hematocrit.

Cultures were maintained synchronized. This was done using the percoll-sorbitol method. Essentially, 80% percoll-sorbitol (in 10xRPMI-HEPES) and 40% percoll-sorbitol (diluted in incomplete media) was prepared. This was then layered on a gradient, with the cells and centrifuged at 2000rpm for 10 minutes, without brakes, resulting in 3 cell layers. The uppermost layer contained debris or dead cells and was aspirated out. The middle layer contained the schizonts and the pellet contained RBCs and other stages of the parasite. This allowed collection of schizonts and other parasite stages separately and these were further washed twice with complete media and used for subsequent experiments.

Occasionally, synchronization using 5% sorbitol (in distilled water) was also done. This method consisted of spinning down the total volume culture and adding 10x volume of 5% sorbitol to the pellet. This was then placed in an incubator at 37°C for 12 minutes and further spun down at 1000rpm for 2 minutes. The sorbitol was then aspirated, and the pellet was washed once with complete media and placed back in culture.

*Mosquito infection and sporozoite preparation*
To infect mosquitoes, gametocyte cultures were maintained in RPMI media and later fed to mosquitoes. On day 16 of the culture, the gametocyte culture was spun down for 5 minutes at 2200 rpm and combined with fresh human blood. The *anopheles* mosquitoes were kept in cardboard cups covered with a mesh netting and a water-bath circulation containing glass feeders was prepared. The water-bath was kept at 37°C, allowing the blood to maintain warm. Parafilm was placed around the glass membrane feeder (mimicking the skin of a human) and placed faced down on the mosquito cup. This was held together using clamps and gametocyte containing blood was added to the feeder. Mosquitoes were allowed to feed for about 30 minutes and once they have fed, the system was shut down and properly decontaminated. To the infected mosquitoes, a sugar meal was provided (cotton with 10% sucrose changed daily) and incubated for 7 days (62).

Then, salivary gland dissection was performed under the light microscope. The mosquitoes were first anesthetized at 4°C, rinsed with 70% ethanol and 1X PBS. Dissection was done in Hanks’ balanced salt solution (HBSS). Following dissection, the salivary glands were crushed to release the sporozoites and further counted using a hemocytometer count (63).

**Protein expression in the sexual and asexual stages**

**Immunofluorescence assay (IFA)**

Immunofluorescence assay (IFA) was performed to characterize the localization of the RON11 protein in different stages of the parasite lifecycle. IFAs were done for both the blood stage (rings, trophozoites, and schizonts) and the mosquito stage (sporozoites), using the RON11 protein obtained from the mice immunization protocol explained above. Many rhoptry proteins are secreted into the newly forming parasitophorous vacuole during merozoite invasion of RBC.
Upon cytochalasin D (cytoD) treatment that blocks the actin motor, merozoites attach, form the junction and secrete their rhoptry contents into the RBC (Ref). Therefore, we examined if RON11 may be secreted in a similar way. Late stage schizonts were purified by percoll-sorbitol gradient and allowed to rupture and invade fresh RBCs in the presence of 2µM cytoD.

IFA is based on 1) binding a specific antibody to a protein of interest and 2) tagging this antibody with a fluorescent dye so that it can be visualized by microscopy (64). The protocol is the same, independent of the stage of the parasite. It essentially consists of a fixation step, permeabilization, primary and secondary antibody incubation sandwiched between a few washing steps and finally, mounting and visualization. First, infected cells were gently spread over a two-circle microscopic slide. Once the cells dried, they were fixed. Initially, different fixation methods were tested. These included fixation with ice-cold 100% methanol, ice-cold acetone and with Paraformaldehyde (PFA) (2.5% PFA and 0.01% GA in 1% sucrose 1x PBS). Both methanol and acetone appeared to be highly dehydrating for the samples and mostly PFA fixation method was used. Once the cells have dried, PFA was added directly to the slide and incubated for 10 minutes. This was then removed and let to air-dry. Once air-dried, the slides were permeabilized using blocking buffer (1x PBS, 2% albumin and 0.1% Triton x 100) for 1 hour at room temperature. This allows permeabilization of the extracellular membrane and will consequently allow the subsequent antibodies to access the inside of the cell. After cells were permeabilized, the primary antibody was added and incubated overnight at 4°C. For this step, multiple antibodies were assessed. First, anti-RON11 rat was used with a 1:50 dilution. This was co-localized with AMA1, as a positive control, using 1:500 dilution. After obtaining the correct dilution for the RON11 antibody, it was co-localized with MSP1 (1:100), RAP2 (1:100) and CSP (1:500). As these are
markers for different organelles within the parasite, it allowed us to assess the correct localization of the RON11 protein, throughout different stages of the lifecycle. The following day, the slides were washed 5 times using washing buffer (1x PBS, 0.1% Triton x 100) for 3 minutes each. The slides were then incubated for 2 hours in secondary antibody at room temperature. Depending on the primary antibody used, either anti-mouse 488, anti-rat 633 or anti-rabbit 633 was used, in a 1:1000 dilution. Subsequently, the slides were washed with washing buffer 5 times for 3 minutes each. Lastly, they were mounted with a drop of mounting media containing ProLong Gold DAPI and covered with a coverslip. The slides were left overnight and sealed with clear nail polish the next day to prevent them from drying out.

DMi8 Leica inverted microscope was used for imaging. The 100x objective was used. Initially, bright light was used to focus the cells and subsequently, fluorescence channels were used to visualize the fluorescent dyes. The DAPI/Blue channel was used to visualize the nuclear stain and the respective fluorescent channels were used to visualize the fluorescence based on the fluorophore attached during the secondary antibody.

Immuno Electron Microscopy (IEM)

Immuno electron microscopy (IEM) was also performed to further classify and analyze the localization of the RON11 protein. This was done for both the asexual and sexual stages. For the asexual stages, parasites were tightly synchronized using percoll-sorbitol at a 40-80% gradient. When parasites were in mature schizont phase (segmentors), these were then spun down and washed twice with PBS. For sporozoites, about 100 mosquitoes were dissected and the salivary glands were collected. Salivary glands were also washed twice with PBS. Samples were fixed by
resuspending the pellet in 4% PFA in HEPES buffer (0.25M, pH 7.4) for 1 hour at room temperature. After an hour, the samples were spun down at 3,000 rpm for 10 min and further resuspended in 8% PFA in HEPES buffer (0.25M, pH 7.4) and left overnight at 4°C. These were then sent to Yale University for further analysis. Due to processing time, these results have still not yet been obtained.

**Western Blot**

Synchronized *P.falciparum* parasite culture was used to purify schizonts. A percoll-sorbitol was performed, in which a gradient of 80% sorbitol (in 10xRPMI-HEPES), 40% sorbitol (diluted in incomplete media), and the cells were assembled. This was centrifuged at 2000rpm for 10 minutes, without brakes. The schizont and pellet were collected and further washed twice with complete media (RPMI/HEPES medium with 5% Albumax). The schizont layer was then lysed using 10% saponin and PMSF (1uM). The lysing buffer was added to the schizonts and mixed properly, incubated on ice for 2 min and then centrifuged at 5000 rpm for 3 min for about 3 times or until a clear pellet was obtained. This depended on the number of cells collected. Subsequently, the pellet was washed 3 times with 1x PBS at 5000 rpm for 3 min. The pellet obtained was then used for SDS-PAGE, by diluting it in 30ul LDS and reaction buffer, boiling at 95°C for 10 min, centrifuged at 10000rpm for 10 min and further running the supernatant on a bis-tris gel using MES buffer. The gel was transferred using the same procedure explained above. The primary antibody used was anti-RON11 rat 1:500 dilution, followed by Goat anti-rat IgG (H+L)+HRP 1:1000 dilution.
**Parasite neutralization assay**

Neutralization assay was performed to evaluate the activity of anti-RON11 antibodies against *P. falciparum* parasites. First, a buffer exchange was performed to transfer the IgG into incomplete RPMI. The IgG was concentrated using Amicon Ultra-4 10kD centrifugal filter. Protein was concentrated to ~ 20mg/mL and then sterile filtered using a Millipore Ultrafree 0.22 micron centrifugal filter. Parasite culture was synchronized using a 10% sorbitol solution, incubated 13 min at 37°C and washed in complete media. Once this synchronized culture matured to late trophozoites, the cells suspension was adjusted to 0.5% parasitemia and 4% hematocrit were incubated with serially diluted anti-RON11 IgGs (14.5, 4.8, 1.6, 0.5 and 0 mg/ml) in a 96-well plate. These samples were incubated at 37°C, cells were collected after 72 hours and stained with SYBR Green I. Parasite growth was measured using a flow cytometer.

**Sporozoite motility assay**

To study the possible role and localization of RON11 on the sporozoites, a sporozoite motility assay was performed (65, 66). First, the *P. falciparum* infected mosquitoes were dissected, and the salivary glands were collected in 1X PBS or HBSS. These were then crushed to release the sporozoites and further counted using a hemocytometer count.

The motility assay was done in and 8-chambered Lab-Tek well, which were initially coated with PBS and 2% Bovine serum albumin (BSA) for 1h at 37°C. BSA allows formation of a coat for sporozoites to undergo gliding. BSA (0.2%) was also added to the sporozoites and incubated for 20 minutes, to induce gliding. The sporozoites were then added to the chamber, spun down at 500rpm for 10 minutes and left for about 30 minutes at 37°C. Blocking buffer (1x PBS, 2% BSA,
0.1% Triton-100) was added and left to incubate for 1h at 37°C. After an hour, the blocking buffer was removed, and the samples were fixed using ice-cold 3% PFA in PBS, for 10 minutes. Following fixation, 3 washes were done for 5 minutes, using wash buffer (PBS, 0.1% Triton-100). The primary antibody was then added and left for 1h at 4°C. The primary antibodies used were anti-RON11 rat 1:25 and 1:50, anti-CSP mouse 1:500, anti-AMA1 mouse 1:500. The following day, the wells were washed using wash buffer 3 times for 5 minutes and the secondary antibodies were added for 2 hours. The secondary antibodies used were anti-mouse 488 1:1000 and anti-rat 633 2:1000. Following incubation, the wells were again washed and then mounted using a drop of mounting media containing ProLong Gold DAPI and covered with a coverslip. The slides were left overnight and sealed with clear nail polish the next day to prevent them from drying out. These were visualized using DMi8 Leica inverted microscope, as explained above.

**Genetic manipulation of RON11**

**Generation of conditional knockout (cKO) RON11 plasmid**

A plasmid was designed that contains a 1.5 kb homology arm followed by a Sera2 gene intron containing a loxP site (IntLoxp), a re-codonized PfRON11 c-terminal containing the EF hand domains, TetR aptamer, and a 3’UTR followed by a second loxP site. Homologous integration of the plasmid is mediated either via random or Crispr-mediated double stranded DNA break. The resulting modified locus will be amenable to conditional protein expression (using anhydro tetracyclin- TetR-DOZI system), or conditional knockout using rapamycin-induced excision at the LoxP sites in parasites expressing dimerizable Cre recombinase (diCre) resulting in a 319 amino acid deletion of the RON11 c-terminus (67-69). This system essentially consists of two fragments of Cre recombinase fused to FRB and FKBP domains respectively. Upon addition of rapamycin,
the FRB and FKBP domains come together reconstituting a function Cre enzyme which recognize
the two 34 bp loxP sites to mediate recombination, resulting in excision of the sequence between
them (63, 64).

This plasmid was designed in a step-by-step approach. First, the pKD-PfAUBL plasmid available in
the laboratory, was double digested using the restriction enzymes XmaI and Ascl at 37°C
overnight. This backbone contained a TetR-DOZI, which allows for control of gene expression. A
gene fragment containing the two PlntLoxp locuses, and 3x HA tag and a 10x Aptamer was
synthesized. This region had a 20bp overhang on both sides, which allowed for gibson assembly
to insert it into the pKD-PfAUBL backbone. Once it was inserted, a single digestion with Ascl was
performed for 3 hours at 37°C, which allowed for insertion of the RON11 homologous
recombination (HR) regions by gibson assembly. The RON11 HR region 1 and 2 were assembled
by PCR. The PCR reaction contained the master mix CloneAmp, PfDNA, MiliQ water and primers
1 and 2 and primers 3 and 4 (see appendix A for primer sequences). The PCR products were
confirmed by agarose gel electrophoresis and purified using Monarch DNA Gel Extraction kit.
Once the two regions were assembled, they were then pieced together using PCR containing
primers 1 and 4 (see appendix A for primer sequences). This was further PCR amplified with
primers 5 and 6 to add the 20bp overhang for gibson assembly (appendix A). This was then run
on an agarose gel and purified using the Monarch DNA Gel Extraction kit and further sanger
sequenced.

Once confirmed insertion of the RON11 HR region into the plasmid backbone, the plasmid was
digested with restriction enzyme BamHI overnight at 37°C. A RON11 EF-hand domain was
synthesized, which contained the 20bp overhangs, allowing it to be inserted by gibson assembly,
flanked by the PIntLoxp. The final product resulted in a plasmid with the Aptamer, RON11 C-terminus and the RON11 EF-KO domain (Figure 9A).

For all the gibson assemblies performed, the NEBuilder HiFi DNA Assembly Protocol from New England BioLabs was followed, as explained above.

This plasmid was transformed in XL-10 Gold Ultracompetent Cells and selected on Kanamycin positive LB agar plates. Colonies were selected and cultured in LB Broth containing 100ug/mL kanamycin overnight at 37°C on a 225rpm shaker. The plasmids were then purified using ZymoPure Plasmid Miniprep or Midiprep kit and concentrations were measured on a NanoDrop 1000 Spectrophotometer. Lastly, these were verified by sanger sequencing.

**Generation of gRNA plasmid**

In addition to the PMG-LoxP-RON11 plasmid, four different guide RNA (gRNA) plasmids were constructed (Figure 9B). This facilitates the induction of double-strand breaks (DSB) in the WT DNA, possibly allowing our PMG-LoxP-RON11 plasmid to be used as a template for DSB recombination and further integrating it into the genome. This will further allow us to study the role of the RON11 KO and the RON11 EF KO, using both the TetR-DOZI and the DiCre-LoxP mechanism (67-69).

Initially, the sgRNA oligos were phosphorylated and annealed, using the oligomers, ligase buffer and T4 PNK kinase. This step was performed on a thermocycler, with the following conditions: 37°C, 30 min, 94°C, 5 min and rampdown at 5°C /min to 25°C. The PCasg backbone was digested using the restriction enzyme Bsal at 37°C for 3 hours. This was then run on an agarose gel to verify digestion, purified using the DNA Gel Extraction kit and further dephosphorylated.
Dephosphorylation was done using the 1x Thermo Fast AP reaction buffer and 10u FastAP alkaline phosphatase at 37°C for 1 hour. Once the oligomers were annealed and phosphorylated, they were diluted 1:200 and the ligation reaction was done using 10x ligase buffer and T4 ligase enzyme. For this reaction, 50ng of the Pcasg backbone vector was used and it was left incubating at 1 hour at room temperature. The plasmid was transformed in XL-10 Gold Ultracompetent cells and selected on Amp positive LB agar plates. A few colonies were selected for each gRNA and inoculated in LB broth containing 100ug/mL Amp overnight at 37°C on an incubator-shaker at 225rpm. These were then purified using ZymoPURE Plasmid Miniprep kit or Midiprep kit and the concentration was measured using a NanoDrop 1000 Spectrophotometer. The plasmids were then confirmed by sanger sequencing.
Transfections

Genetic alterations in *P. falciparum* is a long process, as transfection efficiency is very low due to the high A/T-richness of the genome. For parasite transfection, either rings or mature schizonts can be used (67-69). We have attempted both.

The NF54-DiCre parasites were used for transfection of the PMG-LoxP-RON11 plasmid. These parasites contain a DiCre cassette (novel NF54-DiCre parasites ref). As previously described, upon addition of rapamycin, Cre recombinases are heterodimerized and may recognize the LoxP sites on the plasmid, mediating excision and consequently removing the gene of interest.

The gRNAs would induce a DSB in the parasite genome, allowing the parasites to use the homology template in the plasmid for homology-directed repair (70).

Both ring stage and mature schizont stage transfections were attempted to integrate the plasmid DNA. For both, parasites were tightly synchronized using percoll-sorbitol on a 80%-40% gradient, with the culture layered on top. This resulted in a schizont layer which could be collected and a
pellet with the rings and trophozoite stages. The parasites were placed back in culture. When cultures reached ~5-10% parasitemia transfection was done.

For the mature schizont stage, schizonts were purified on the day of transfection using percoll-sorbitol. Schizonts were then gently mixed with plasmid DNA and P3 buffer (final volume of 100ul). This mixture was then electroporated with the Lonza Nucleofactor 4D using the P3/CM162 program in cuvettes. The cuvette was then incubated at 37°C for 30 minutes and then placed in a culture flask with pre-warmed complete media with RBCs at 10% hematocrit. The flask was placed on a shaker at 37°C for 20 minutes and further diluted to 4% hematocrit and incubated at 37°C, 5% CO₂. The following day, drug pressure was applied in the media.

In addition, ring stage transfections were also performed. For this, 100ul of either P3 or cytomix (120mM KCl, 0.15mM CaCl₂, 8.66mM K₂HPO₄, 1.36mM MgCl₂, 25mM HEPES, 2mM EGTA, pH 7.6) was used and 4ul of ATP (100mM) was added. The plasmid DNA was then resuspended in this reaction. The packed RBCs containing rings were washed with cold cytomix and the pellet was further resuspended in DNA containing solution. This was then electroporated in cuvette on Lonza Nucleofactor 4D using the P3/CM-150 program. The cuvette was then incubated at 37°C for 30 minutes and the cells were then transferred to a 15mL centrifuge tube that contained 5mL of warm media and centrifuged at 1000rpm for 7 minutes. The pellet was then resuspended in 2.5mL pre-warmed media and transferred to a 6 well plate and incubated at 37°C and 5% CO₂. The following day, appropriate drug pressure was applied.
Results

*In silico* identification of RON11 as a candidate calcium sensing protein in *P. falciparum* merozoites and sporozoites

We conducted an *in silico* search in PlasmoDB for potential calcium binding proteins (CaBPs) that may be involved in rhoptry secretion. Our search was limited to *P. falciparum* proteins containing the two most common calcium binding domains, namely, the EF-hand and C2 domains. We identified 62 proteins (excluding rifin and emp1 gene family) containing either EF-hand or C2 domain or both. We searched for proteins with strong evidence (at least 2 unique peptides identified by mass spectrometry) of expression in mature schizonts or merozoites. This yielded 29 proteins. Since rhoptry secretion is required for successful parasite invasion, we hypothesized that any CaBP involved in this process is likely to be essential. Analyzing data from the piggyback insertion screen identified 15 out of 29 to be likely essential (mutagenesis index score <0.4) in *P.falciparum* (72-74). Besides merozoites, the only other form of the parasite requiring rhoptry function is the sporozoite. Hence, CaBPs functioning in rhoptry secretion is also likely to be expressed in this stage. Analyzing the mass spectrometry data from proteins expressed in sporozoites identified 7 of 15 to be expressed in both merozoites and sporozoites (Table 1).

*Table 1.* Table listing mass spectrometry data of calcium-binding proteins expressed in sporozoites and merozoites.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Product Description</th>
<th>Function in asexual stages</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF3D7_0217500</td>
<td>calcium-dependent protein kinase 1</td>
<td>Invasion</td>
<td>(75-77)</td>
</tr>
<tr>
<td>PF3D7_1025000</td>
<td>Eps15-like protein</td>
<td>Endocytosis</td>
<td>(78)</td>
</tr>
</tbody>
</table>
Our searches interestingly identified the double C2 domain protein previously shown to be required for microneme secretion (84), while some others have been implicated in invasion (CDPK1), endocytosis (Eps15-like), ER calcium sensing (PfERC), cytosolic calcium sensor calmodulin and PfRON11, a multimembrane domain protein containing a pair of EF hands whose function is not known in asexual stages. Recently, a PfRON11 homolog in P. berghei was shown to be required for sporozoite invasion of hepatocytes (40, 50). However, its function in blood forms is not characterized. Interestingly PbRON11 appears to localize to the rhoptry in both sporozoite and merozoite (40, 50). This positioned PfRON11 as a prime candidate for a calcium sensing and rhoptry secretion.

RON11 – Domain architecture, homology model & EF-hand; Expression profile (essentiality curve from plasmoDB, mRNA, protein)

RON11 structure consists of an N-terminal signal peptide, seven transmembrane domains, and two C-terminus putative calcium-binding EF-hand domains (Figure 10A) (36). Interestingly, our recent quantitative mass spectrometry identified PfRON11 to be higher abundance in P. falciparum invasive compared to non-invasive merozoites, suggesting a role in invasion (50). In
addition, it has been identified as an essential protein, having a length of 1071 amino acids (72-74). The protein has been shown to be expressed in both sexual and asexual stages of the parasite lifecycle (40, 50).

The predicted EF-hand domains were further analyzed by homology modeling using Phyre2 (85). Indeed, this region of RON11 aligned better with the EF-hand domain present in PfCDPK2 (PDB: 4MVF) and was used to model the predicted calcium binding sites (Fig 11). The sequence of the loop regions between the E and F helices were predicted to bind 4 Ca2+ ions (Fig 11).
**Figure 10.** In silico characterization of RON1 (72-74). (A) Represents the essentiality curve, depicted on plasmoDB, indicating that it is an essential gene (72-74). (B) Bar graph comparing RON11 levels in long-lived merozoites (LLM) and non-invasive merozoites (control). There is a significance increase in RON11 levels in long-lived merozoites, supporting that RON11 may have an essential role in invasion (111). (C) Depicts the structure of RON11. The protein contains a signal peptide on the N-terminus, seven transmembrane domains and two EF-hands at the C-terminus. The amino acid sequences used for the plasmid generation are also represented below.

**Figure 11.** The figure depicts the homology-based modeling of PiRON11 using Phyre2 software and the sequence of the RON11 EF-hand below.
RON11-MBP fusion protein purification and Ab generation in Rats

Protein expression and purification

RON11-MBP protein expression was induced using IPTG, the cells were lysed and run on an SDS-PAGE to verify protein expression. As the protein contains a (His)6-tag, verification could be done using anti-His primary antibody. The western blot showed expression of the intact RON11-MBP fusion protein (expected size 58.3kDa) in the induced but not in uninduced sample (Fig 12A and B). The protein was first purified using FPLC on an IMAC column followed by clean-up using a ceramic hydroxyapatite column (CHT) column (Fig 12C and D). Following purification, protein concentration was determined using A280 and fractions that detected protein were run on an SDS-PAGE (Fig 12C). From the FPLC graphs, we detected that the protein was relatively pure, as we just detected one prominent peak as expected (Fig 12C). However, a few additional bands likely corresponding to some E.coli proteins were also detected. Therefore, the protein was further purified using mixed resin ceramic hydroxyapatite (CHT) column chromatography, 0.2mL fractions were collected and analyzed by SDS-PAGE (Fig 12D). Enriched fractions were pooled for subsequent dialysis against phosphate buffered saline (PBS) and aliquots stored in -80°C.
Figure 12. RON11-MBP protein expression and purification. (A) Coomassie gel staining of total protein from *E. coli* after IPTG induced protein expression. Uninduced *E. coli* was used as negative control. (B) Western blot analysis of *E. coli* expressed RON11 using mouse anti-His primary antibody and anti-mouse-HRP conjugated secondary antibody. (C) Depicts RON11-MBP protein purification using the IMAC column. The FPLC graph is shown on the left and the right depicts the SDS-PAGE of the elution protein fractions. (D) RON11-MBP protein purification using CHT column. Left panel shows a typical chromatogram showing a single peak and the right panel is the SDS-PAGE analysis of proteins from the FPLC fractions, eluted fractions. IRON11-MBP fusion protein was detected using mouse anti-His antibody and anti-mouse-HRP secondary antibody. The expected molecular weight of the RON11-MBP fusion protein is 58.3 kDa.
**RON11 expression in *P. falciparum* schizonts**

Analysis of gene expression data indicates that RON11 transcription is upregulated late during the intra erythrocytic cycle, specifically in the schizonts stage (72-74). Late stage schizonts were enriched on a percoll-sorbitol gradient and total protein lysate was run on SDS-PAGE, transferred onto PVD membrane. Western blot analysis with anti-RON11 antibody shows the presence of a specific high molecular weight band along with two additional low molecular weight band (Fig 13). This may be suggestive of protein processing and its importance in parasite biology needs to be evaluated.

*Figure 13.* Western blot showing the expression of RON11 protein in *P.falciparum* mature schizonts. The expected molecular weight of the RON11 protein is 128 kDa. The antibody used to detect RON11 is the rat anti-RON11.
Next, immunofluorescence microscopy was performed to determine the localization of RON11 within the parasite. To do this we co-labeled schizonts with anti-RON11 antibody and markers of microneme (AMA1), rhoptry (RAP2) or parasite surface (MSP1). AMA1 is a micronemal protein and appears as a puncta within the merozoites of intact schizonts. RON11 signal also appears to be punctate suggesting apical organelle localization. However, the signal for AMA1 and RON11 clearly do not overlap (Fig 14A). Interestingly, in the extracellular merozoite, AMA1 re-localizes to the surface as seen by a peripheral distribution while RON11 is still apically localized (Fig 14A inset). This suggests that either the function of RON11 is confined to the rhoptry or it may be secreted during invasion after interaction with host RBC. The apical staining of RON11 was further confirmed when co-labeled with surface protein marker MSP1 (Fig 14B). However, co-labeling with rhoptry bulb marker RAP2 show clear areas of overlap with RON11 signal (Fig 14C). This suggests that RON11 may be stored in or near the rhoptry bulb, in agreement with the localization of the *P.berghei* RON11 homolog (72-74).

Immunofluorescence microscopy of invasion-arrested merozoites show a distinct apical localization of RON11 overlapping with RAP2 in extracellular merozoites and merozoites interacting with RBC (Fig 15A-C). Interestingly, in the cytoD arrested merozoites, RON11 appears to be secreted into the RBC and overlaps with the RAP2, a rhoptry protein known to be secreted (Fig 15D and 20E). This indicates that perhaps RON11 is secreted along with the rhoptry contents in vacuoles after tight junction formation.
Figure 14. Immunofluorescence assay (IFA) depicting RON11 protein localization in the asexual stages. (A) AMA1 and RON11 protein localization in mature schizonts. (B) MSP1 and RON11 protein localization in mature schizonts. (C) RAP2 and RON11 protein localization in mature schizonts.
Figure 15. Merozoites were treated with cytochalasin D to block invasion of the parasites into new RBCs, allowing visualization of the protein localization during tight junction formation by Immunofluorescence assay (IFA). The RON11 and RAP2 antibodies were used to assess protein localization. Additionally, a schematic representation is shown that depicts the respective stage of the parasite.
**Antibody activity**

Topology predictions suggest that the N-terminus of PfRON11 may be accessible if the protein is secreted on to the parasite surface during invasion and thus accessible to antibodies. Hence, we evaluated the neutralizing activity of the antibodies targeting RON11 N-terminus. Serum from rats immunized with recombinant RON11 protein was used to purify total IgG using Protein G columns. The purified IgG was concentrated, and buffer exchanged into incomplete RPMI media. Standard neutralization assays were performed in the presence of varying concentration of IgG and the neutralizing activity of the anti-RON11 antibody was measured by flow cytometry. This assay measures the increase in parasitemia following 2 rounds of invasion. Our results demonstrate moderate neutralizing activity exhibited by the antibodies targeting RON11 N-terminus, suggesting that this region of the protein may be exposed briefly during invasion (Fig 16).

![Neutralizing activity of anti-RON11 antibodies](image)

*Figure 16. Neutralizing activity of anti-RON11 antibodies. The ability of anti-RON11 polyclonal antibody targeting the N-terminus of RON11 to neutralize *P. falciparum* 3D7 strain was assessed by flow cytometry.*
**RON11 expression in *P. falciparum* salivary gland sporozoites**

Besides merozoites, the only other life cycle stage of *Plasmodium* containing rhoptries are the sporozoites. Therefore, we examined RON11 expression in *P. falciparum* sporozoites collected from salivary glands of infected female *Anopheles* mosquitoes. RON11 labeling stained sporozoites exhibit a punctate labeling similar to micronemal and rhoptry markers AMA1 and RAP2 respectively (Fig 17A and 17C). A punctate localization suggestive of secretory organelle localization was also confirmed by co-labeling with CSP, a sporozoite surface antigen (Fig 17B). Ongoing immune electron microscopy studies will determine the precise localization of RON11 in the sporozoites.
As RON11 was secreted by merozoites during invasion, we reasoned that it might also be secreted during sporozoite motility. A motility assay was also performed to determine if RON11 is secreted during sporozoite motility (Fig 18). Sporozoites were allowed to glide on albumin coated chamber slides for 30min at 37C. Samples were fixed and labeled with anti-AMA1 and anti-RON11 antibodies and imaged by fluorescence microscopy. While AMA1 was confined to the sporozoites, RON11 signal appear to indicate protein secreted as evidenced by a wide distribution of punctate labeling around the area of the sporozoites. Additional motility assay
together with the well-establish CSP protein known to be secreted will be needed to confirm these observations.

![Figure 18](image1.png)

*Figure 18. Immunofluorescence assay (IFA) depicting AMA1 and RON11 protein localization during sporozoite motility.*

**RON11cKO construct and transfections**

To study the function of RON11 in *P. falciparum* blood stages, a plasmid knock out construct incorporating the dimerizable cre-loxP system was generated. As described in the methods section, this construct was made in a step-by-step approach. First the pIntLoxP cassette, 3x HA tag and 10x Aptamer were cloned into the pKD-PfAUBL backbone. RON11 homologous recombination region was assembled by PCR and cloned into this plasmid backbone. A re-codedonized optimized fragment corresponding to the C-terminus RON11 EF-hand was cloned between the loxPInt and the HA tag. Figure 19A shows the agarose gel images for the final plasmid (11465bp).

Additionally, four gRNA plasmids were constructed to facilitate transfections by induction of a double stranded break (DSB) into the Figure 19B depicts the sanger sequencing results, which indicate that the gRNAs were cloned into the plasmid and have the correct expected sequence.
These were further used for transfections, as previously explained. Table 2 describes the different plasmid concentrations and drug pressures applied for each transfection condition. However, due to the efficiency of these transfections and despite multiple attempts, no transfection was performed successfully.

![Image](image_url)

*Figure 19.* Generation of plasmids to genetically modify *P.falciparum* parasites. (A) Plasmids ran on an agarose gel to check purity of the plasmids for transfections. (B) Depiction of sequencing results for gRNA4, showing a perfect match with the plasmid.

*Table 2.* LoxP-PMG-RON11 and gRNA transfection conditions

<table>
<thead>
<tr>
<th>Transfection (schz or rings)</th>
<th>Buffer</th>
<th>DNA conc (ug)</th>
<th>gRNA conc (mg/ml)</th>
<th>Drug pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schz</td>
<td>P3</td>
<td>10ug</td>
<td>gRNA 3 – 10ug</td>
<td>Anhydrotetracycline (ATC – 2.5mM) and Blasticidin (BSD – 2.5mg/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gRNA 4 – 5ug</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>11ug</td>
<td>gRNA3 – 10ug gRNA4 – 10ug</td>
<td>ATC (2.5mM), BSD (2.5mg/ml) and DSM1 (1.8mM)</td>
</tr>
<tr>
<td>--------</td>
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<td>------</td>
<td>---------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Schz</td>
<td>P3</td>
<td>9ug</td>
<td>gRNA3 – 8ug gRNA4 – 6ug</td>
<td>ATC (2.5mM) and BSD (2.5mg/ml)</td>
</tr>
<tr>
<td>Rings</td>
<td>P3</td>
<td>9ug</td>
<td>gRNA3 – 8ug gRNA4 – 6ug</td>
<td>ATC (2.5mM) and BSD (2.5mg/ml)</td>
</tr>
<tr>
<td>Rings</td>
<td>Cytomix</td>
<td>10ug</td>
<td>gRNA3 – 3ug gRNA4 – 3ug</td>
<td>ATC (2.5mM) and BSD (2.5mg/ml)</td>
</tr>
<tr>
<td>Rings</td>
<td>Cytomix</td>
<td>10ug</td>
<td>gRNA1 – 3ug gRNA2 – 3ug</td>
<td>ATC (2.5mM) and BSD (2.5mg/ml)</td>
</tr>
<tr>
<td>Rings</td>
<td>Cytomix</td>
<td>10ug</td>
<td>gRNA3 – 3ug gRNA4 – 3ug</td>
<td>ATC (2.5mM) and BSD (2.5mg/ml)</td>
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<tr>
<td>Rings</td>
<td>Cytomix</td>
<td>10ug</td>
<td>gRNA1 – 3ug gRNA2 – 3ug</td>
<td>ATC (2.5mM) and BSD (2.5mg/ml)</td>
</tr>
</tbody>
</table>

**Expression of recombinant RON11 EF domain**

As described earlier, the C-terminus of RON11 contains a pair of predicted EF hand domains (Fig 20). A mammalian codon-optimized DNA fragment coding for the EF hand domains with (His)6 tag was synthesized and cloned into the pcDNA3.1 plasmid and sequence verified (Figure 20A). The plasmid was transfected into HEK293T cells. Protein expression was analyzed in both the cell
pellet and culture supernatant. We also tested protein expression using the TnT SP6 Wheat Germ Protein Expression system (Figure 20B and 20C). Coomassie staining of total protein did not clearly show a specific protein. Therefore, western blot analysis using anti-His antibody was performed. A 28kD protein corresponding to the expected size was identified in the soluble fraction of HEK293 cell lysate (Fig 20C). These experiments also identified the optimal amount of plasmid needed for transfections. The western also detected a high molecular weight band, likely a mammalian protein cross reacting with the anti-His antibody. Recombinant protein will be purified from ongoing large-scale transfection, purified by IMAC using FPLC and used to evaluate its ability to bind calcium.

Figure 20. PcDNA3.1-RON11 EF-hand protein expression and purification. (A) PcDNA3.1 (1) and PcDNA3.1-RON11 (2) plasmid ran on an agarose gel to assess purity for transfections. (B) Coomassie to detect protein expression of the samples. 1-Neg ctrl insoluble cell pellet, 2-Neg ctrl lysate, 3-RON11 1ug insoluble cell pellet, 4-RON11 2ug lysate, 5-RON11 2ug insoluble cell pellet, 6-RON11 2ug lysate, 7-RON11 4ug insoluble cell pellet, 8-RON11 4ug lysate, 9-TnT SP6. (C) Western blot probing his-tag to detect RON11 EF-hand protein expression. 1-Neg ctrl insoluble cell pellet, 2-Neg ctrl lysate, 3-RON11 1ug insoluble cell pellet, 4-RON11 2ug lysate, 5-RON11 2ug insoluble cell pellet, 6-RON11 2ug lysate, 7-RON11 4ug lysate, 8-RON11 4ug insoluble cell pellet, 9-TnT SP6. The expected size for the RON11 EF-hand is 28 kDa.
Discussion

Malaria has been around for many years and continues to have a devastating impact worldwide, having about 229 million cases worldwide in 2019 (1-3). Some countries, in particular westernized countries, have managed to eradicate the disease; however, other countries continue to have high disease prevalence and deaths due to the malaria parasite (World Malaria Report, 2020). Nonetheless, children under 5, pregnant women, and travelers to malaria-endemic countries are the most affected by the severity of the disease (2, 5, 6, 86). Although it was first identified in 1880 and has been studied since then, there is still a gap in the complexity of the parasite lifecycle and in the development of vaccines to prevent disease (2, 3). Currently, multiple prevention strategies are implemented such as the use of insecticides, IRS, and ITNs. Further, chemotherapy and chemoprophylaxis have also been developed to prevent and treat disease and symptoms (2, 3, 6, 10, 12, 15-17, 86). One major concern is that the malaria parasite is highly polymorphic and can easily find strategies to overcome its prevention and treatment methods. Additionally, these methods are highly costly and require external efforts that involve political and social contributions, making implementation slightly challenging (10, 44, 45, 86). Currently, vaccines are being studied, targeting the pre-erythrocytic or erythrocytic stage or even blocking the transmission of the parasite (6). Ideally, these vaccines would induce an immune response that targets a protein or proteins essential for parasite invasion and lifecycle progression, blocking the cycle and hampering replication and thus, disease. Some mechanisms to block the progression of the lifecycle, include parasite motility, invasion, egress, or hepatocyte traversal ability. More recently, conserved proteins present in different stages of the parasite lifecycle are being evaluated as vaccine candidates, leading to the possible development of a multi-stage vaccine.
This will hopefully allow overcoming parasite immune evasion strategies and developing a vaccine with high efficacy (87, 88). Nowadays, with an increased political instability and climate change, this facilitates diffusion of the parasite to countries that do not have incidence of cases, and thus, developing prevention strategies, not only help prevent severe disease but also aid in limiting spread of the disease (5, 6).

The goal of this study was to characterize the role of P.fRON11 in \textit{P.falciparum} merozoites and sporozoites. First, we attempted to express RON11 antibodies that would allow to characterize the protein. First, mice were immunized against this protein, raising antibodies that could be used for further studies. With the antibodies, we were able to characterize protein localization in both the sporozoite and schizont blood stages and immunoblotting to detect expression in the parasites. Further, we attempted to conditionally knock out the protein and assess how the lifecycle progresses when the protein is knocked out. In addition, this protein contains an EF-hand, a calcium-binding domain, which could be essential in signaling processes required for lifecycle progression. Thus, we developed a plasmid line to allow the expression of the EF-hand in mammalian cells. This could then be further purified and used to study calcium binding by using fluorescence assays. By characterizing the protein, it can further be evaluated as a possible vaccine candidate.

We were able to express the RON11-MBP fusion protein in bacterial cells, purify it using the IMAC columns and the CHT purification method. CHT appeared to be the purest product, and this was then used for rat immunizations. With the antibodies obtained, we were able to detect using a GIA that indeed the antibodies inhibit invasion of the parasites, having an IC50 of 10.67 mg/ml.
A limitation of this study is that we were unable to cleave the MBP fusion protein after the purification of the protein, as the RON11 would lose its stability. MBP is larger in size when compared to RON11, making it difficult to assess the number of antibodies raised for RON11 and thus could affect future studies. Additionally, an enzyme-linked immunosorbent assay (ELISA) should be performed to quantify the number of species-specific antibodies and thus allowing us to quantify the number of antibodies specific to RON11 (64, 89, 90). Further, it would be interesting to do an immunoprecipitation followed by mass spectrometry, giving us a better understanding of the specificity of the antibodies. Furthermore, an essential control for the IFA data would be staining the samples with anti-MBP antibodies, as the RON11 is fused to the MBP protein.

The antibodies were then used to study the localization of the RON11 protein in both the sporozoite and schizont blood stages. We were able to detect that the RON11 protein does not co-localize with AMA1 in the mature schizont stages. AMA1 is localized in the merozoite apex in late-schizonts (91). Further, RON11 does not co-localize with MSP1 and RAP2, which are merozoite surface protein and rhoptry proteins respectively. This indicates that possibly RON11 is localized in the merozoite cytosol. When looking at the sporozoite stages, RON11 appears to have a punctuate pattern. However, it also does not co-localize with AMA1 or CSP (sporozoite surface protein). It does appear to co-localize with RAP2, indicating it is localized in the rhoptries. According to previous studies done on P.berghei, RON11 expression was found to be mainly in rhoptry organelles in merozoites and sporozoites (40, 47). A sporozoite motility assay was also performed to study whether RON11 protein is secreted when motility is induced. In our studies, we were unable to draw conclusive results from this. We used AMA1 as a negative
control, as this protein is not secreted during motility. However, we did see some sort of pattern surrounding the sporozoites, indicating possible secretion. These studies must be repeated using the CSP antibody, which can be used as a positive control. The CSP antibody is secreted during motility and forms trails surrounding the sporozoite. A reason for these inconclusive results could be that the sporozoites were only incubated with 2% BSA (to induce motility) for 10 minutes, not giving them enough time to glide.

For the co-localization experiments performed, quantitative data should be obtained to allow for more conclusive results. Additionally, different antibodies should be used such as the rhoptry neck protein 4 (RON4) to better characterize the protein localization. Additionally, super resolution microscopy should be performed to allow for better resolution and thus better localization studies of the protein. Ongoing immuno-electron microscopy (IEM) is being performed to determine the exact localization of the protein in both schizonts and sporozoites.

Additionally, a western blot was done on mature schizonts (segmentors), in which the expression of RON11 protein was detected. Surprisingly, we detected 3 different bands for the RON11 (15kDa, 35kDa and 70kDa) and we were expecting a band at 128kDa. This could indicate that the protein undergoes some sort of processing in this stage of the parasite. It would be interesting to do a western blot on the different stages of the schizonts, from less mature to more mature schizonts, to detect if this pattern is consistent throughout the development of the parasites. Additionally, it would be interesting to test different protease inhibitors to study if this processing is a result of biological processing or technical errors that might have occurred.
Despite multiple transfection attempts, we were unable to generate *P. falciparum* RON11 conditional knockouts and obtain a phenotype for this. Transfection efficacy in *P. falciparum* parasites is very low (67-69). For future studies, it would be interesting to test the selection linked integration (SLI) method. It essentially consists of tagging the gene of interest with a T2A skip peptide and a selectable marker (e.g. Neomycin) at its C-terminus. When the plasmid is integrated, the endogenous target fuses with the tag and parasites can be selected using neomycin. When integrated, the target gene is disrupted (92, 93).

Additionally, we were able to construct a plasmid that contains the PcDNA3.1 backbone and the RON11 EF-hand protein. This plasmid can further be used for transfection in HEK293T cells and protein purification of the EF-hand protein. After purification, the protein can further be used for fluorescence assays, to detect Ca2+ binding to the protein (94-96).

RON11 is indeed a promising vaccine candidate, as it is expressed in both the sexual and asexual stages of the parasite lifecycle and appears to be essential for invasion of the parasites. Targeting calcium signaling could block multiple downstream effector pathways that the parasite would need to invade and progress its cycle (95, 96). If RON11 is found to play an important role in mediating calcium signaling in the parasites, it would be interesting to test if antibodies to RON11 will disrupt this process.
Part II: Development of a chimeric mouse model to study next-generation malaria vaccines
Introduction

AMA1-RON2 interaction

AMA1 is a conserved protein among different *Plasmodium* species that are found to be essential for invasion. It translocates from the micronemes to the merozoite surface during invasion, where it binds to RON proteins in the MJ (26, 43-45, 97). In addition to its expression in the merozoites, PfAMA1 is also expressed in the sporozoites and has been shown to be essential for hepatocyte invasion (23, 24).

It is initially produced as an 83-kDa precursor protein, which undergoes processing and is cleaved. This results in releasing an N-terminal prodomain, resulting in a 66-kDa protein. The protein contains 16 cysteine residues, which form disulfide bonds that fold and produce the three extracellular domains of AMA1. Additionally, it contains a hydrophobic pocket formed by two PAN domains, and this is the region where interaction with RON proteins occurs (figure 4) (26, 43-45, 97). RON2 protein is translocated into the host cell and takes a membrane-spanning conformation (97). In addition, it has been shown to have 2 to 4 hydrophobic helices that directly interact with AMA1 (15). More specifically, RON2 is composed of a conserved region referred to as RON2L, which is thought to be essential for AMA1 interaction (15, 43-45).

AMA1-RON2 interaction: a target of neutralizing antibodies

Malaria-exposed individuals present high antibody titers against AMA1 (43). The AMA1-RON2 interaction has been studied as an antimalarial target and has been considered a leading blood-
stage vaccine candidate (15, 43-45). A study done by Srinivasan et al. demonstrated that vaccinating rats with a PfAMA1-RON2L complex induced invasion inhibitory antibodies. The neutralizing antibody levels were higher in the PfAMA1-RON2L complex than compared to vaccination with AMA1 alone (43). Moreover, they vaccinated nonhuman primates with PfAMA1-RON2L complex and further challenged them with a virulent P.falciparum FVO strain and observed nearly 90% vaccine efficacy (43). This demonstrates that this essential protein-protein interaction could be a good target for a malaria vaccine.

An issue that appears in developing blood-stage vaccines is that many proteins exhibit polymorphism, likely due to immune selection and vaccination against these proteins does not raise sufficient amounts of functional antibodies targeting all polymorphisms (15, 43-45). It is now thought that targeting a small number of alleles could be sufficient to account for the polymorphisms (15, 43-45). Regarding the number of functional antibodies produced, it is thought that targeting the PfAMA1-RON2L complex could overcome this challenge (43-45).

Models to study vaccine targets and PPIs

AMA1 vaccines have been tested in vivo in mice, rats, and Aotus monkeys (15, 43-45, 98). In evaluating vaccine candidates and efficacy, animal models are helpful to assess protection against challenge, dosage, delivery route, correlates of protection, and type of immune response elicited (99). However, deciding on which animal model to use can be challenging. Outbred large animal models such as nonhuman primates are often the best model, as they best represent humans (99, 100). However, using such models is costly, requires adequate infrastructure and
training, and a more stringent ethical authorization due to high level of emotional development of the primates (99, 100). Additionally, this model still lacks immunological tools to characterize the immune response, which is an important feature to assess when considering vaccine candidates (99, 100).

Although nonhuman primate immunization with AMA1 has shown to elicit an immune response, human trials using AMA1 vaccines have shown not to be as effective, as the number of functional antibodies produced is not sufficient to induce immune protection of the human host (43-45). Additionally, current studies of AMA1 vaccines are strain-specific, making it harder to assess the efficiency of the vaccine and possible antigenic polymorphisms that may occur in *P. falciparum* human malaria (15). Different models are infected by different malaria parasite species (100). For example, the *Aotus* monkeys are not naturally infected with *P. falciparum* human malaria parasites, and this can account for the differences observed in human AMA1 trials.

Mouse malaria models have been used to study vaccine candidates and the protective immune response elicited. These are less costly and easier to manipulate. However, they can only be challenged with mouse infecting parasites, such as *P. berghei* and *P. yoelii*. In addition, there is variation in the rodent and human genome as well as in antigens and proteins (100, 101). Nowadays, using genetic approaches, it is possible to engineer murine models to mimic human immune responses by creating transgenic mouse models that contain human receptors.
Aim

The aim of this project was to optimize transfection of *P.yoelii* to develop a chimeric *P.yoelii* mouse model expressing different forms of the human *P.falciparum* AMA1 gene. The goal of this is to develop an *in vivo* chimeric model to evaluate next-generation vaccine designs, using rodent parasite expressing functional *P.falciparum* proteins. Plasmid constructs encoding full length and processed forms of PfAMA1 under the control of *P.yoelii* AMA1 regulatory elements were generated. Culture conditions and nycodenz density gradient purification of *P.yoelii* schizonts were optimized and transfections with Py/PfAMA1 chimeric constructs were performed.

Methods

Plasmid Design

This project was based on the novel Gene Insertion Marker Out (GIMO) protocol. The *P.yoelii* XNL GIMO motherline was obtained from Drs. Photini Sinnis and Shahid Khan. This motherline contains the hdhfr:yfcu drug marker integrated in the 230p genomic locus. The GIMO method essentially consists of insertion of transgenes by removal of the drug marker. When transfections are performed, the construct can recombine, and the marker is removed, and the gene of interest is inserted. This is enhanced by negative selection using the 5-fluorocytosine (5-FCU) drug (Figure 1) (102, 103).
Two different plasmids were constructed, one containing the full-length AMA1 83-kDa molecule and another one with the AMA1 66-kDa processed form. These plasmids contained the \textit{P.yoelii} AMA1 promoter and the \textit{P. falciparum} AMA1 gene (Figure 2).

First, the 230p 3′ and 5′ ends were assembled by PCR reaction using primers 7 and 8 (see appendix A). These were then pieced by gibson assembly and further cloned into the pDEF-\textit{HR} plasmid backbone. The pDEF-\textit{HR} backbone was first digested using HindIII restriction enzyme at 37°C for 3 hours. This was then run on an agarose gel and purified using Monarch DNA Gel Extraction kit and phosphorylated using the Fast Alkaline Phosphatase enzyme for 30 min at 37°C and 75°C for minutes to deactivate the enzyme activity.

\textit{Figure 1.} Schematic representation of the GIMO motherline, outlined in the red box (97).
After assembly of the 230p locus into the backbone, the plasmid was digested using XbaI at 37°C for 3 hours. The *P.yoelii* and *P.falciparum* AMA1 were assembled together by PCR. This fragment contained a 20bp overhang for the 230p and was further inserted by gibson assembly. These were then transformed into XL-10 Gold Ultracompetent cells, colonies selected and purified, as explained above. The plasmids were then confirmed by a double digestion using KpnI and HindIII restriction enzymes and by sanger sequencing.

Once confirmed, the plasmid was double digested using KpnI and HindIII, to linearize the 230p-PyAMA1-PfAMA1 region. This was run on a gel, purified and DNA precipitated, to be used for transfections. DNA precipitation was done by addition of 1/10 volumes of acetate and 2.5 volumes of 100% ice cold ethanol. This reaction was mixed and left at -20°C overnight. The following day, the sample was centrifuged at 13000rpm for 30 minutes, the ethanol was poured off and the pellet was washed 2x with 70% ethanol. The pellet was then left to air dry and further resuspended in cytomix buffer to be used for transfections.

![Plasmid map of Py230PyAMA1-PfAMA1 83 kDa (A) and 66 kDa (B).](image)

*Figure 2.* Plasmid map of Py230PyAMA1-PfAMA1 83 kDa (A) and 66 kDa (B).
**P.yoelii transfection protocol and optimization**

For the transfection protocol, we followed the protocol published by Jongco et al in 2006 (102, 103). As this protocol had never been used in our lab, multiple optimization steps were required (protocol outlined in figure 3).

Initially, mice were infected with *P.yoelii* GIMO motherline (parasitemia >3%). The parasites were thawed at room temperature and passaged into 2 swiss-webster mice intraperitoneally (i.p). The parasites were positively selected using pyrimethamine drug for 4 days, in order to select for parasites containing the *hdhf::yfcu* marker. After 8 days of injection, mice had a parasitemia of

*Figure 3. Schematic representation of P.yoelii transfection protocol.*
~6% and a cardiac puncture was done under anesthesia in a heparin-containing tube. With the blood collected, new aliquots were made in 30% glycerol-PBS and stored at −80°C.

For transfections, 4 mice were infected with the *P. yoelii* GIMO motherline frozen stock. Initially, mice were directly injected by i.p, however, this took about 2-3 weeks for them to reach a parasitemia of ~15%, which is required for transfection and schizont purification. We then tested if injecting the mice with 200ul Phenylhydrazine (Phz) (6.3ul of phz stock in 1mL PBS) would accelerate the rate of parasitemia of the mice, as Phz induces reticulocyte formation, and this parasite strain preferably invades reticulocytes. Injecting the drug 48h prior to challenge indeed accelerated parasitemia rate, having ~15% parasitemia at about ~1.5 weeks post challenge. After challenge, mice parasitemia was monitored daily.

When the parasitemia reached 15-20%, a cardiac puncture under anesthesia was done and about 1mL of blood was collected per mouse into heparin-containing tubes. The blood was washed in complete culture medium (RPMI 1640 medium containing 20% heat inactivated fetal calf serum and 25mM Hepes) by centrifugation at 300xg for 8 min. Then, 30mL of CM per 1mL of blood was added and the culture was incubated at 37°C at 10% O₂ and 5% CO₂ on a shaker at 77rpm for 8 hours. The culture was monitored hourly from hour 4 onwards, allowing the parasites to mature and reach segmentor stages.

Once cultures compromised of majority of mature schizonts (segmentors), schizonts were purified. Multiple methods of schizont purification were tested. The standardized method, as described in the protocol, consisted of layering 35mL of the culture onto 10mL of 60% nycodenz in PBS and further centrifuged at 200xg at room temperature with no breaks, resulting in a layer of schizont and a cell pellet with remaining stages. This method, however, was not as effective in
our lab and alternative methods were tested. Different nycodenz percentages were tested, such as 55%, 65% and 70%, with 65% showing the best efficiency in schizont purification (Figure 4). In addition to nycodenz-based purification, the standard percoll-sorbitol method was also tested. This method is typically used in *P.falciparum* cultures to purify schizonts. This consists of preparing a gradient of 70% and 40% Percoll-sorbitol and overlying the parasite solution and centrifugating this at 10,000rpm for 20 minutes with no breaks, resulting in 3 layers. The uppermost layer comprises of debris, the middle layer of the schizonts and the pellet with remaining parasite stages or uninfected cells (Figure 13). The last method tested for schizont purification was magnetic purification. This consists of using MACS Separation Columns. The column was pre-wet with CM and further the sample was loaded. The valve was opened slightly, allowing the sample to slowly pass through the magnetic column and the schizonts to bind to the magnet, as they contain larger amounts of the haemozoin crystal and were further eluted (Figure 4) (104, 105).

*Figure 4. Different schizont purification methods used. A) Nycodenz, B) Percoll-sorbitol and C) Magnetic purification.*
Following schizont purification, transfections were done using the Amaxa Human T cell Nucleofactor kit. Essentially, 100ul of the AMAXA nucleofactor solution was added to about 5ug of DNA. This mixture was used to resuspend the schizont pellet \((5\times10^7)\) and transferred to the cuvette, where electroporation occurred using pre-programmed settings on AMAXA nucelofector. After transfection, 400ul of PBS was added to the sample and about 200ul was injected into mice by tail vein injection (i.v). These new mice recipients were previously injected with Phz drug. At 24h post injection, appropriate drug treatment was started.

With regards to drug treatment, the 5-FCU drug was used, which allowed for a negative selection of the parasites by killing all parasites expressing \(yfcu\), allowing for parasites that underwent gene integration to survive. Two different drug administration routes were tested. Mice were first injected by i.p with 20mg/ml of the drug for four consecutive days. Mice were then monitored until they reached about 8% parasitemia and blood was then collected to confirm insertion (described below). As this requires large concentration of drug and is a more invasive procedure, we also tested administering the drug in drinking water of mice \((106)\). We compared administration of 5-FCU by i.p \((20\text{mg/mL})\) and by drinking water \((0.75\text{mg/mL})\) for four consecutive days. As the latter appeared to be as efficient, this was the preferred method used for drug administration.

**Confirmation of transfectants**

Following appropriate drug treatment, mice underwent cardiac puncture under anesthesia. The DNA from the blood was extracted using the DNAeasy Blood & Tissue kit, the concentration was measured using a NanoDrop 1000 spectrophotometer and further used for PCR confirmation.
The PCRs were performed using primers specific for the *P.falciparum* AMA1-66 and AMA1-83 (appendix A, primers 9, 10, 11, 12, 13).

**Results**

**Plasmid construction and digestion**

Multiple attempts were performed to construct the plasmids, as the PyAMA1 and PfAMA1 gene is relatively large (approximately 8000bp). This was done on a step-by-step basis. First, the 230p 3´ and 5´ end were cloned into the pDEF plasmid backbone. In parallel, the PyAMA1 and PfAMA1 66 and 83kDa were pieced together via PCR. Following plasmid backbone digestion and PyPfAMA1 construction, these were cloned into the backbone, giving rise to two different plasmid constructs. Confirmation of the inserts was initially done by double restriction digestion and run on an agarose gel, where two bands were expected. Figure 5 shows the agarose gel image, which depicts a band for the plasmid backbone (5276bp) and insert (PfAMA1 66 – 2941bp and PfAMA1 83 – 3143bp). Lastly, the whole plasmid was confirmed by sanger sequencing.
P. yoelii transfections protocol optimization

In a first attempt to do the *P. yoelii* transfections, the protocol was followed exactly as described (107). Three mice were infected and the whole blood was collected via cardiac puncture at a parasitemia of 6% (Figure 6A). These were incubated for 8 hours with CM containing FBS to allow schizont maturation, prior purification. Purification was done using a nycodenz gradient of 55% and 60%. In this attempt, schizont purification did not occur and no clear separation was seen, however, as parasites were mainly in segmentor stage, we proceeded with the transfections without purification. For transfection, 1μg of DNA was used for PfAMA1-66 and 1μg of DNA for PfAMA1-83 and infected two mice, one for each transfection. One of the mice (PfAMA1-66) died during i.v injection, perhaps due to injection of air bubbles. For the mouse that survived, we

Figure 5. Gel image depicting PfAMA1-66 and PfAMA1-83 plasmids.
initiated the 5-FCU drug treatment 4 days post-transfection, when parasites became visible via giemsa smear. The drug treatment was done via i.p for four days. During drug treatment, the parasitemia decreased and following drug treatment, there was an increase in parasitemia, as expected (Figure 6B). This indicated that transfected parasites were selected for. A PCR was done to confirm insertion of the PfAMA1-83 gene using primers 7 to 11 (appendix A). Both the PfAMA1-66kDa and PfAMA1-83kDa primers were used, as controls. From the PCR result, it appeared that this transfection was positive and thus, blood was collected, and new mice were re-infected and further undergone drug treatment. For the re-infected mice, the PCRs appeared negative (figure 6C). This was probably due to the fact that we only started the drug treatment when parasites became visible and allowed WT parasites to replicate and clear out transfected parasites.
Figure 6. *P. yoelii* transfections. (A) Outline of protocol used for transfection. (B) Giemsa smear of transfected mice at day 1 and day 3 pre-drug treatment, day 6 and 7 during drug treatment and day 12 and 14 post-drug treatment. (C) Agarose gel which shows PCR results done to detect if *PfAMA1-83* was integrated.
The *P.yoelii* transfection protocol required multiple optimization steps. It was found that injecting the Phenylhydrazine (Phz) drug 48h prior challenge, accelerated infection and increase of parasitemia of the mice. Initial infections would take 2-2.5 weeks for the mice to reach a parasitemia of ~10%, however, with the drug injection, it took only 1-1.5 weeks for the mice to reach ~20% parasitemia. Higher parasitemia is desired for *P.yoelii* transfections, as it allowed for a better schizont purification.

The protocol followed found that schizont purification was optimal using a nycodenz gradient of 60% (106). However, when this was tested in our lab, we were unable to get a clear separation of the schizonts. We then attempted different purification methods such as different nycodenz gradients (60%, 70%, 75%), histodenz (55%) percoll-sorbitol (70% and 40%) and, magnetic purification (Fig 7). The percoll-sorbitol method was not optimal in purification and a mixed stage of parasites was obtained (Fig 27B). Regarding the nycodenz, the 65% and 70% gradient were the only ones that gave us a schizont separation. When comparing both of the gradients, the latter seemed to provide more number of segmentor schizonts and less of a mixed culture (Figure 7A and C). The magnetic purification also allowed for parasite purification, however, different schizont maturities were purified and not solely segmentors (Figure 7D). With this we found that 70% nycodenz gradient would be the most optimal for schizont purification.
Lastly, a study by A. Waters in 2012 compared administration of the 5FCU drug via i.p injections or in the water of mice for four days (106). We then decided to infect mice with WT GIMO parasites and start a 4 day drug treatment, where we treated one group of mice with 5-FCU via i.p injections (10mg/ml) and another group by administering the drug in the drinking water of mice (0.75mg/ml). The parasitemia is plotted in figure 8. This showed us that there was no major difference in administering the drug via i.p or drug water.
Following all these attempts, we were able to find optimal conditions for the transfections. In summary, we found that 1) injecting the mice with Phz drug 48h prior to infection, allowed for a faster increase in parasitemia, 2) Schizont purification was optimal using 70% nycodenz, 3) Drug should be administered 24h post-transfection even if no parasites are identified and 4) Drug can be administered via drinking water.

In addition, we discussed with Dr.Kaneko’s group in Japan that also works on *P.yoelii* transfections. Their transfection protocol had a few modifications. They did not incubate the schizonts for 8 hours to allow them to mature and instead, collected the schizonts at a strategic timepoint. Additionally, a smaller amount of CM was added to the infected RBCs, making them more concentrated. We attempted this as well.
**P. yoelii optimized transfection**

Following optimization, transfections were attempted with the ideal conditions. For this, mice were injected with Phz drug 48h pre-infection. They were infected with 200ul of WT GIMO parasites and about 1 week later, they reached a parasitemia of ~15%. The blood was collected by cardiac puncture and pooled. It was then washed with 30ml of ICM and spun down at 2100rpm for 5 min at RT. The supernatant was removed and 6ml of CM containing FBS was added. The mature schizonts were purified using 70% nycodenz in PBS and spun down at 2500rpm for 20 minutes at RT, without brakes. The schizonts were collected and washed once with ICM. After counting with a hemocytometer, $10^8$ schizonts were used per transfection. Three transfections were done, as described in table 1. For this attempt, only the PfAMA1-83kDa was transfected and both the linear and circular plasmid were tested.

*Table 1. PfAMA1 transfection conditions.*

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<th>Parasites</th>
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<tbody>
<tr>
<td>Mock – no plasmid</td>
<td>100ul</td>
<td>$10^6$</td>
</tr>
<tr>
<td>pDEF- PfAMA183 kDa circular plasmid (10ug)</td>
<td>100ul</td>
<td>$10^6$</td>
</tr>
<tr>
<td>pDEF- PfAMA183 kDa linear plasmid (1ug)</td>
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<td>$10^6$</td>
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</table>
Following electroporation, the samples were diluted in 150ul of PBS and injected into the mice by i.v. The following day, the mice were treated with the 5-FCU drug via drug water for four consecutive days, at a concentration of 1.5mg/ml. The parasitemia was monitored daily and a decrease in parasitemia was observed for all mice. Three days post-removal of the drug, an increase in parasites were observed for all mice, however, there was a more prominent increase in the mice transfected with the PfAMA1-83kDa plasmids. The blood was collected by cardiac puncture and the DNA was extracted using the DNAeasy blood & tissue kit and further, a PCR was done to detect if there was insertion (primers 7,8,9,10,12,13,14,15 were used, appendix A). Despite the parasitemia of the mice increasing, the PCRs indicate that the transfections were negative and insertion of PfAMA1 did not occur, as no band is detected when using PfAMA1-83kDa specific primers (Fig 9).
Discussion

In this second study, we worked on optimizing a protocol for the generation of a chimeric *P. yoelii* mouse model expressing different forms of the human *P. falciparum* AMA1 gene. AMA1 has previously been studied as an essential gene required for invasion. It has been shown that AMA1 binding to RON2L allows for the formation of the moving junction and further invasion of the merozoites into the RBCs (43-45). In addition, studies have been done in our lab that vaccinated *Aotus* monkeys against the AMA1-RON2L complex, showing a protective response (43-45). However, parasite proteins have been shown to have species-specific differences and before proceeding with clinical trials against AMA1-RON2L, further evaluation of the *P. falciparum* AMA1-RON2L gene is required *in vivo*. Thus, the second aim of this project was to develop a chimeric *P. yoelii* mouse model expressing different forms of the human *P. falciparum* AMA1 gene.

*Figure 9.* Agarose gel which shows PCR results done to detect if PfAMA1-83 was integrated.
As the transfection process is relatively new, multiple optimization steps were required. Due to lack of time, in this project, we limited ourselves to optimizing the transfection protocol in an attempt to insert the *P. falciparum* AMA1 gene.

After attempting the transfection using the optimized protocol, we were still unable to confirm integration of PfAMA1 by PCR. Perhaps maintaining the mice under drug treatment for longer than four days would allow the WT parasites to be eliminated from the mice blood and allow for better selection transfected parasites. If this allows for better detection and selection of transfected parasites, the next step would be to undergo limiting dilution. This consists of diluting the cloned parasites so that only a single method is used to infect multiple mice. These mice are then screened and assessed. Mice containing solely mutants are used for further screening (108, 109).

In future studies, it would be interesting to generate a *P. yoelii* mouse model containing both the *P. falciparum* AMA1-RON2L genes. This model would allow better assessment of next-generation vaccines *in vivo* using *P. falciparum* genes.
References


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## Appendix A: Table of Primers

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<th>Primer number</th>
<th>Primer name</th>
<th>Primer sequence</th>
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